

Open Research Online

The Open University's repository of research publications and other research outputs

The association of Clostridium perfringens with foal diarrhoea

Thesis

How to cite:

Netherwood, Trudy (1995). The association of Clostridium perfringens with foal diarrhoea. PhD thesis The Open University.

For guidance on citations see [FAQs](#).

© 1995 The Author



<https://creativecommons.org/licenses/by-nc-nd/4.0/>

Version: Version of Record

Link(s) to article on publisher's website:

<http://dx.doi.org/doi:10.21954/ou.ro.0000e0d4>

Copyright and Moral Rights for the articles on this site are retained by the individual authors and/or other copyright owners. For more information on Open Research Online's data [policy](#) on reuse of materials please consult the policies page.

oro.open.ac.uk

THE ASSOCIATION OF
Clostridium perfringens
WITH FOAL DIARRHOEA

BY

TRUDY NETHERWOOD

A thesis submitted in partial fulfilment of the requirements
of the Open University for
the degree of Doctor of Philosophy

March 1995

Animal Health Trust,
P.O. Box 5,
Newmarket,
Suffolk,
CB8 7DW

- work supported by
The Home of Rest for Horses and Lloyds of London.

Authors no: P 9272080
Date of submission: March 1995
Date of award: 11th July 1995

1.0 SUMMARY

Several case reports of *Clostridium perfringens* involvement in equine enteric disease have not identified the prevalence and statistical association of these bacteria with foal diarrhoea. Each of five methods which favoured the recovery of *C. perfringens* in different physiological states were chosen to improve the sensitivity of isolation in a survey of foal diarrhoea for *C. perfringens* and other pathogens.

C. perfringens was significantly associated with foal diarrhoea (isolated from 57% of 421 scouring animals but from only 33% of 222 controls; odds ratio 7.4; $p < 0.001$ by multivariate analysis); it was also associated with fatal diarrhoea (odds ratio 2.7; $p = 0.047$). Rotavirus, *Cryptosporidium* sp. and large numbers of *Strongyloides westeri* were the only other pathogens associated with diarrhoea although they were less prevalent than *C. perfringens*; *Salmonella* sp. was the only other pathogen associated with fatal diarrhoea.

Enterotoxin production was detected by reverse passive latex agglutination test (RPLA) amongst isolates of *C. perfringens* from scouring and healthy foals. The enterotoxin gene from an equine strain was cloned and its sequence was essentially identical to that published for a human isolate. Less than 5% of *C. perfringens* isolated from scouring foals and 0.5% from controls were positive for the enterotoxin gene by polymerase chain reaction (PCR) (odds ratio 19.1; $p < 0.005$). Presence of the enterotoxin gene was confirmed in representative isolates with a gene probe of chromosomal DNA and PCR product as well as neutralisation of cytotoxicity by anti-toxin. Enterotoxigenicity of half of RPLA positive isolates could not^{be} confirmed in this way.

Enterotoxigenic *C. perfringens* were a probable cause of foal diarrhoea. However, a greater proportion of the disease was associated with non-enterotoxigenic *C. perfringens*. There is now a need to identify molecular differences between non-enterotoxigenic *C. perfringens* strains from scouring and healthy foals which might be associated with pathogenicity.

5.1.2 List of Figures.....	35
5.2 Introduction	36
5.2.1 The study of foal diarrhoea and <i>C. perfringens</i>	36
5.2.2 The isolation and identification of <i>Clostridium</i> <i>perfringens</i>	36
5.2.2.1 Cultural and biochemical characteristics of <i>C. perfringens</i>	36
5.2.2.2 Culture of <i>C. perfringens</i> endospores.....	41
5.3 Objectives	46
5.4 Methods and materials.....	47
5.4.1 Bacteria	47
5.4.2 Media and culture conditions	47
5.4.3 Samples of foal faeces	48
5.4.4 Pre-treatment of cultures or faecal suspensions.....	48
5.4.5 Identification of <i>C. perfringens</i>	48
5.4.6 Enumeration of endospores and viable <i>C. perfringens</i>	48
5.5 Results and Discussion	50
5.5.1 The effect of different pre-treatments and culture conditions on the recovery of endospores.	50
5.5.2 Isolation of <i>C. perfringens</i> from foal faeces by different methods.....	51
6.0 Chapter Three - A microbiological survey of foal diarrhoea.....	56
6.1 Contents.....	57
6.1.1 List of Tables.....	57
6.2 Introduction	59
6.2.1 Microbiology of foal diarrhoea.....	59
6.3 Objectives	61
6.4 Methods and Materials	62
6.4.1 Collection of faecal samples from foals.....	62

6.4.2 Detection of Pathogens	62
6.4.3 Statistical Analyses	65
6.5 Results and Discussion	69
7.0 Chapter Four - The cloning and genetic characterisation of <i>Clostridium</i>	
<i>perfringens</i> enterotoxin from equine isolates	77
7.1 Contents.....	78
7.1.1 List of Tables.....	79
7.1.2 List of Figures.....	79
7.2 Introduction	80
7.2.1 Virulence factors of <i>Clostridium perfringens</i>	80
7.2.2 The <i>Clostridium perfringens</i> enterotoxin.....	80
7.3 Objectives	82
7.4 Methods and Materials	83
7.4.1 Enterotoxin detection.....	83
7.4.2 Bacteria	84
7.4.3 Media	84
7.4.4 Purification of <i>C. perfringens</i> DNA.....	85
7.4.5 General molecular methods	85
7.4.6 Agarose gel electrophoresis.....	85
7.4.7 SDS-PAGE.....	86
7.4.8 PCR amplification of a putative enterotoxin gene	
fragment from an equine strain of <i>C. perfringens</i>	87
7.4.9 Cloning of the putative enterotoxin gene into plasmid	
p <i>Trc</i> 99 A.....	88
7.4.10 Detection of recombinant p <i>Trc</i> 99 A	89
7.4.11 Induction of enterotoxin gene expression	90
7.4.12 Single stranded DNA sequencing	90
7.4.13 Statistical Analyses.....	95
7.5 Results and Discussion	96

8.0 Chapter Five- Detection of the <i>Clostridium perfringens</i> enterotoxin gene	
in equine isolates and its association with foal diarrhoea	102
8.1 Contents.....	103
8.1.1 List of Tables	103
8.1.2 List of Figures.....	103
8.2 Introduction	104
8.3 Objectives	105
8.4 Methods and Materials	106
8.4.1 Polymerase chain reaction	106
8.4.2 Enterotoxin gene probe.....	106
8.4.3 Statistical Analyses	107
8.5 Results and Discussion	108
9.0 Chapter Six - Discussion.....	113
9.1 Contents.....	114
9.2 Discussion.....	115
9.2.1 Isolation of <i>Clostridium perfringens</i> from foal faeces	115
9.2.2 Detection of <i>Clostridium perfringens</i> and other	
pathogens in association with diarrhoea	117
9.2.3 Enterotoxin and equine <i>Clostridium perfringens</i>	122
9.2.4 <i>Clostridium perfringens</i> as a cause of foal diarrhoea	125
10.0 References.....	132

2.1 List of Tables

Table 1. Recovery of <i>Clostridium perfringens</i> NCTC 8239 from a sporulated culture of different ages treated at 80°C for different times (% of total endospore count before treatment).....	53
Table 2. Recovery of <i>Clostridium perfringens</i> NCTC 8239 from a sporulated culture of different ages treated at 60°C for different times (% of total endospore count before treatment).....	53

Table 3. Recovery of <i>Clostridium perfringens</i> NCTC8239 from a sporulated culture of different ages treated with 50% ethanol at 25°C for different times (% of total endospore count before treatment).....	54
Table 4. Mean recovery of <i>Clostridium perfringens</i> NCTC8239 from 22 hour sporulated cultures by different endospore stimulation methods (percentage of total endospore count before treatment).....	54
Table 5. Isolation of <i>Clostridium perfringens</i> from 271 equine faeces by five different methods.....	55
Table 6. Distribution of samples between Thoroughbred and other foals at stud or other premises.....	67
Table 7. Distribution of samples between scouring and healthy foals of different ages.....	67
Table 8. Distribution of the duration of scouring in foals before the faecal sample was collected.....	67
Table 9. Distribution of samples between scouring and healthy foals from different parts of the country.....	68
Table 10. Frequency of detection of <i>Strongyloides westeri</i> >2000 oocysts per gram of foal faeces.....	68
Table 11. Adjusted frequency of detection of <i>Strongyloides westeri</i> >2000 oocysts per gram of foal faeces.....	68
Table 12. Detection rates of pathogens in faeces of scouring and healthy foals.....	73
Table 13. Primary multivariate analysis of the association of pathogens with foals with diarrhoea compared with healthy foals not-in-contact with diarrhoea cases.....	73
Table 14. Final multivariate analysis of the association of pathogens with foals with diarrhoea compared with healthy foals not-in-contact with diarrhoea cases.....	74
Table 15. Multivariate analysis of the association of pathogens with foals with diarrhoea compared with all healthy foals.....	74

Table 16. The effect of different <i>C. perfringens</i> isolation methods on multivariate analysis of the association of pathogens with diarrhoea	74
Table 17. Univariate analysis of the numbers of <i>C. perfringens</i> cfu's per gram of faeces isolated from foals with diarrhoea and healthy foals not-in- contact with cases of diarrhoea	75
Table 18. Rate of detection (%) of pathogens in foals with a history of diarrhoea and which subsequently died (n=22).	76
Table 19. Age distribution of the isolation of <i>C. perfringens</i> from scouring foals which died	76
Table 20. Analysis of Rotavirus, <i>Clostridium perfringens</i> , and <i>Cryptosporidium sp.</i> in different parts of the country.	76
Table 21. Analysis of the interactions between <i>C. perfringens</i> and rotavirus	76
Table 22. Detection of enterotoxin by RPLA directly in faeces and in sporulated culture supernatant fractions of isolates of <i>C. perfringens</i>	101
Table 23. Detection of the enterotoxin gene by Polymerase Chain Reaction from <i>C. perfringens</i>	110
Table 24. Reaction of isolates positive or negative by RPLA in the PCR, gene probe against chromosomal DNA extract, gene probe against PCR product and verotoxicity neutralised by anti-enterotoxin serum	111
Table 25. Univariate analysis of the numbers of <i>C. perfringens</i> cfu's per gram of faeces isolated from foals not-in-contact with cases of diarrhoea	112
Table 26. Univariate analysis of the association of PCR positive <i>C. perfringens</i> with large numbers of cfu's per gram of faeces ($\geq 10^4$) isolated by three different methods	112

2.2 List of Figures

FIGURE 1. Distribution of the number of <i>C. perfringens</i> isolations from foal faeces by direct isolation methods (n = 271)	55
FIGURE 2. Distribution of the number of <i>C. perfringens</i> isolations from foal faeces samples by enrichment methods (n = 271)	55

FIGURE 3. Comparison of distribution of the number of <i>C. perfringens</i> isolations from foal faeces samples by direct culture and enrichment methods (n = 271)	55
FIGURE 4. Photograph of an agarose gel with PCR products of B3550.5, NCTC8239, and JM105 recombinant containing the enterotoxin gene.	98
FIGURE 5. Photograph of an SDS-PAGE gel of the <i>E. coli</i> JM105 p <i>Trc</i> 99A enterotoxin gene recombinant and non-recombinant culture supernatant fractions.....	99
FIGURE 6. Sequence of <i>Clostridium perfringens</i> B3550.5 enterotoxin gene.....	100
FIGURE 7. Distribution of the number of enterotoxigenic <i>C. perfringens</i> identified by RPLA and PCR methods	110

3.0 ACKNOWLEDGEMENTS

The author would like to thank Lloyds of London and The Home of Rest for Horses for their financial support for this work; Unipath Limited for their subsidy of the cost of the PET-RPLA kits; Dr. Moira Brett at the Central Public Health Laboratory for serotyping many isolates; veterinarians and stud farm managers for kindly taking samples of faeces from foals; James Wood for statistical advice; Dr. Matthew Binns for advice on molecular biological techniques; my supervisors, Neil Chanter and Jenny Mumford, for all their considerable help and advice throughout the project; the members of the Animal Health Trust Bacteriology Section for all their support, in particular, Glenys Hogg for a large amount of the media preparation; and Margaret Blackett, Sarah King, and George Clutterham for processing samples when I was unable (especially when I broke my arm!); and my husband Steve for word processing advice and for all his encouragement throughout the project.

4.0 CHAPTER ONE

PREFACE, INTRODUCTION AND OBJECTIVES

4.1 CONTENTS

4.2 Preface.....	13
4.3 Introduction	14
4.3.1 The mammalian gut - in health	14
4.3.1.1 Normal flora	14
4.3.1.2 Colonisation resistance	15
4.3.2 The mammalian gut - during disease.....	16
4.3.2.1 Infectious agents of diarrhoea	16
4.3.2.2 Comparative aetiology of diarrhoea in mammals	19
4.3.2.3 Treatment and prevention	21
4.3.3 Foal Diarrhoea.....	22
4.3.3.1 Known and possible causes of Foal Diarrhoea.....	22
4.3.3.2 Infectious Foal Diarrhoea.....	24
4.3.3.3 Justification for a study of the rôle of <i>Clostridium</i> <i>perfringens</i> in foal diarrhoea.....	26
4.3.4 <i>Clostridium perfringens</i> as a cause of diarrhoea	27
4.3.4.1 Enterotoxigenic <i>Clostridium perfringens</i>	28
4.4 Objectives	31

4.2 PREFACE

The causes and mechanisms of diarrhoea in mammals will be reviewed against the background of the normal flora and the resistance that it provides to colonisation by pathogens. Surveys and case histories of foal diarrhoea will be discussed in relation to this review. In particular, the potential rôle of *Clostridium perfringens* will be examined to provide the justification for the objectives of this thesis.

4.3 INTRODUCTION

4.3.1 The mammalian gut - in health

The healthy gut is colonised by very large numbers of bacteria of many different genera and species which are collectively known as the normal flora. This gross bacterial infection not only rarely causes disease but also offers resistance to colonisation by pathogenic bacteria.

4.3.1.1 Normal flora

At birth the gut is quickly colonised by a bacterial flora which undergoes a series of changes in its composition. This succession of bacteria depends on environmental factors and the microbial organisms involved (Savage, 1977). The first organisms to colonise the alimentary tract in most species of mammal are *Escherichia coli*, *Clostridium perfringens* and *Streptococcus sp.* followed by *Lactobacillus sp.* and *Bacteroides fragilis*. In man, *Lactobacillus sp.* or *Bifidobacterium sp.* are the first species to colonise breast-fed babies. Once solid food is eaten a wide range of strict anaerobes colonise the gut (Williams Smith, 1965). The initially large numbers of *Clostridium perfringens* in most animal species decline within a few days, with only small numbers remaining after 1 to 3 weeks (Williams Smith & Crabb, 1961). The flora then gradually changes and becomes characteristic for each animal species, for example in the healthy horse, *Clostridium perfringens* is usually only present in lower colonic contents and faeces, but in the dog it is present throughout the gut (Williams Smith, 1965). In humans, early colonisation varies with gestational age, type of delivery and type of feeding (Long & Swenson, 1977). The composition of indigenous flora is primarily modulated by diet and in certain cases may not illicit an immune response, due to oral tolerance and age, (Van der Waaij, 1988) and possibly due to establishment before the host's immunity is mature. Large numbers of lymphocytes (many producing IgA, which has the ability to interfere with pathogenic micro-organisms coming in contact with mucosal surfaces) are spread throughout the inner layers of the intestinal wall as isolated cells or as small cell clusters with larger clusters forming distinct follicles with germinal

centres. It is thought that the immune system may learn to recognise part of the microflora as part of the host organism (Van der Waaij, 1989).

The microorganisms in the gastrointestinal tract are influenced by peristalsis, villous motility, epithelial migration and extrusion, nutrient availability from the host's diet and secretory products, for example, mucins, enzymes, & immunoglobulins, and local conditions including pH, oxidation-reduction conditions, oxygen level, osmolarity, microbial competition, interference or synergy, and the host's antimicrobial systems (Savage, 1977). All these factors influence the initial colonisation of the gastrointestinal tract and maintenance of the normal microbial flora, which in turn affects the host. The gut flora can act as a reservoir of infectious organisms available to take advantage of a decrease in the host's defences, for example to infect wound sites, or cause peritonitis (Drasar & Duerden, 1991). Conversely, it can act to exclude pathogens by interfering with colonisation. Dietary supplementation with antibiotics, such as penicillin and tetracycline in pig and poultry production, in the 1950's, caused a reduction in normal gut flora, which led to a decreased resistance to gut infection (Drasar & Barrow, 1985). This beneficial effect of the normal flora is known as colonisation resistance (Neu, 1988).

4.3.1.2 Colonisation resistance

Important factors influencing colonisation resistance include the maintenance of an anaerobic flora, gut transit time, and production of saliva, mucus and secretory IgA (Van der Waaij, 1987). Facultatively anaerobic and aerobic bacteria are important in scavenging free oxygen, thus maintaining a low Eh and so promulgating the anaerobic flora (Drasar & Duerden, 1991). The microflora once established is relatively stable although certain factors may affect it, albeit transiently. After a meal, for example, a wave of altered bacterial flora passes through the intestine. Antibiotics, however, may disrupt colonisation resistance for longer periods (Tannock, 1983; Van der Waaij, 1987; Borriello, 1992) and in such circumstances in man this may allow *Clostridium difficile* to infect the gut and cause diarrhoea (Borriello, 1992). Colonisation resistance normally prevents *C. difficile* disease.

There may also be a breakdown in regulatory factors in stressed animals so reducing colonisation resistance and allowing easier colonisation by pathogens (Tannock, 1983). Unless absolutely necessary the use of antibiotics should be avoided during diarrhoea to prevent further reduction of colonisation resistance. Probiotics consisting of doses of *Lactobacillus sp.* alone or together with other bacteria, have been used to reinforce colonisation resistance in an attempt to prevent or treat diarrhoea.

The gut flora can, therefore, have both positive and negative effects on the host, but once stable, has an overall positive influence on the host's health through colonisation resistance. Colonisation resistance in the young mammal is not established and the changing microflora is more at risk of challenge by enteropathogens, than in older mammals with a stable microflora. Additionally, in many mammals the active immune system is not mature at birth and colostrum is perhaps the most important protection factor in neonates.

4.3.2 The mammalian gut - during disease

Microbes may be classified as (a) 'pathogens', (b) 'commensals' which are usually found in the normal body flora; and (c) as 'saprophytes' which are normally found in environmental sites such as soil or plants. However, some 'commensals', such as *Escherichia coli* or *Staphylococcus saprophyticus* may act as opportunistic pathogens under certain circumstances. The virulence and pathogenicity of a microbe depends on host as well as on microbial factors. Host factors include the age of the patient, genetic resistance / susceptibility, non-specific immune host defences and local immune and non-immune host defences against infection. There also maybe considerable variation in the possession of virulence factors between strains of the same microbial species or between different species (Shanson, 1982).

4.3.2.1 Infectious agents of diarrhoea

Bacterial enteropathogens of mammals include: *Salmonella sp.*, *Shigella sp.*, *Campylobacter jejuni / coli*, *Campylobacter lari*, Enteropathogenic / Enterotoxigenic / Enterohaemorrhagic *E. coli*, *Vibrio cholerae*, *V. parahaemolyticus*, *Yersinia*

enterocolitica, *Aeromonas hydrophila*, *Plesiomonas shigelloides*, *Clostridium perfringens*, *C. difficile*, *Staphylococcus aureus*, and *Bacillus cereus*. Viral pathogens include: rotavirus, astrovirus, calicivirus, adenovirus, echovirus, parvovirus, coronavirus and Norwalk agent virus. Protozoan enteric pathogens include: *Giardia lamblia*, *Entamoeba histolytica*, and *Cryptosporidium sp.*. Helminthic pathogens include: *Strongyloides westeri*, *Strongylus sp.*, *Trichuris sp.*, *Trichinella sp.*, and *Cyathostoma sp.* to name just a few (Bowman & Rand, 1980; Shanson, 1982; M.A.F.F., 1984).

The healthy gut breaks food into smaller subunits which are absorbed along with water and salts. The layer of mucosal cells lining the gut perform this absorptive rôle. These cells form into enormous numbers of villous projections and each cell membrane facing the lumen has many further microvillous extensions, all of which serve to increase the surface area over which absorption can take place. Interference with absorption in any area of the gut can lead to a lower dry matter content in the gut lumen, and if this is not compensated for in the gut lower down, of faeces also. This is a mechanistic definition of diarrhoea but the extent of this process will further define its clinical significance. Any process reducing microvilli, destroying mucosal cells, interfering with mucosal cell generation, destroying villi, or interfering with the biochemical process of absorption may cause diarrhoea. Furthermore, some pathogens may biochemically stimulate a reversal of absorption which is known as hypersecretion. Otherwise, reduced absorption is known as mal-absorption. In the short term, rapid dehydration may result which is life threatening and longer term disruption to normal gut motility and nutritional absorption can occur before a full recovery is established.

Three major microbial pathogenic mechanisms, adherence, toxin production and invasiveness, play a rôle in diarrhoea, with several mechanisms often acting concomitantly (Robins-Browne, 1985). Also, different pathogens may possess similar virulence traits with the same mode of action and genetic control.

Adherence of some bacteria to the intestinal mucosa seems to be a prerequisite for pathogenic colonisation, enabling organisms to resist expulsion by peristaltic clearing mechanisms accelerated by greater luminal flow rates during diarrhoea. Adherence can then be followed by bacterial proliferation, toxin production or invasion of mucosal cells. These processes have been most extensively investigated in *E. coli*. Adherence is usually mediated by the binding of bacterial surface proteins, named adhesins or lectins, to mammalian cell receptors, which are usually sugar residues. Several adherence-associated surface structures, such as human colonisation factor antigens, porcine K88 antigen and bovine K99 antigen, have been isolated from enterotoxigenic *E. coli* from humans and animals. Adherence itself can sometimes appear to be the sole pathogenic mechanism causing diarrhoea; certain enteropathogenic *E. coli* cause characteristic destruction and effacement of microvilli of the small intestine, thereby causing disease by reducing gut surface area for absorption. Adherence is also an important initial event in the pathogenesis of diarrhoea caused by *Shigella sp.* and *Salmonella sp.*. These bacteria first adhere to the gut surface before invading the mucosa and producing toxins.

Toxins produced by some enteric bacteria are part of a well established pathogenic mechanism in diarrhoea. These enterotoxins act on the mucosal epithelium of the small intestine, causing fluid hypersecretion and profuse watery diarrhoea, without structural damage to the intestinal mucosa. Fluid secretion is caused by the enzymatic effect of enterotoxins on intestinal cells, often through specific receptors and increased intracellular cyclic nucleotide concentrations. Cholera toxin, produced by *Vibrio cholerae*, causes adenosine diphosphate (ADP) ribosylation of an adenylate cyclase regulatory protein, leading to an increase in intracellular cyclic adenosine monophosphate (cAMP). This causes active fluid secretion and inhibition of resorption of salt and water. A functionally similar heat labile enterotoxin is produced by some strains of *E. coli*, *Salmonella sp.*, *Aeromonas hydrophila*, *Bacillus cereus* and *Campylobacter sp.* although the precise biochemical details may vary.

A low molecular weight heat-stable enterotoxin is produced by some strains of *E. coli* which activates guanylate cyclase to increase cyclic guanosine monophosphate (cGMP) concentration, and cause diarrhoea in much the same way as cholera toxin. A similar enterotoxin is produced by *Yersinia enterocolitica*.

Cytotoxins are a further distinct group of potentially diarrhoeagenic toxins. They can kill mammalian cells, usually by inhibition of protein synthesis. Cytotoxic activity can be demonstrated *in vitro* in several tissue culture cell lines. *In vivo* they cause damage to intestinal epithelial cells and interfere with normal absorptive mechanisms. Fluid loss is probably related to impaired absorption since, unlike enterotoxins, cytotoxins apparently do not cause active fluid secretion by the gut nor do they leave the gut undamaged structurally. Shiga toxin is an example of a cytotoxin which is produced by *Shigella dysenteriae* serotype 1. Functionally closely related toxins are also produced by *Clostridium difficile*, *Campylobacter sp.*, *Aeromonas hydrophila*, *Vibrio sp.*, *Plesiomonas shigelloides* and certain serotypes of enterohaemorrhagic *E. coli*.

Invasive bacteria penetrate mucosal cells (*Shigella sp.*) and even the whole mucosa as far as a basal membrane (*Salmonella sp.*), and is the key property of bacteria causing dysentery, which is diarrhoea with frank blood in the faeces. These clinical signs are also seen with diarrhoea caused by *Campylobacter sp.*, enteroinvasive *E. coli*, and *Vibrio sp.*. Other organisms, such as *Plesiomonas shigelloides*, produce clinical and pathologic features that are compatible with invasiveness, but the mechanisms involved and the relationship with other virulence traits, such as toxin production, are currently unclear. Some helminths, such as *Trichinella spiralis*, are invasive to the extent of penetrating the blood and lymph systems to migrate throughout the body.

4.3.2.2 Comparative aetiology of diarrhoea in mammals

Many diarrhoeagenic organisms have parallel pathogenic mechanisms across several species. For example, rotavirus is attributed with 49% of cases of diarrhoea in children (Black, 1985), 20% in pigs (Johnson *et al.*, 1992), 19-72% in calves

(McNulty, 1985; Tzipori, 1985a; Reynolds *et al.*, 1986; Snodgrass *et al.*, 1986), and 8-37% in foals (Conner & Darlington, 1980; Strickland *et al.*, 1982; Tzipori *et al.*, 1984; Tzipori, 1985b; Dwyer *et al.*, 1990; Browning *et al.*, 1991; Holland *et al.*, 1991; Browning *et al.*, 1992). Although several other diarrhoeagenic organisms pathogenic in children, piglets, calves, and lambs have been associated with foal diarrhoea, their significance and prevalence has yet to be determined in a multi-agent survey of a large enough population sample. These include: *Cryptosporidium sp.* (Black, 1985; Tzipori, 1985a; Tzipori, 1985c; Reynolds *et al.*, 1986; Snodgrass *et al.*, 1986); Coronavirus (McNulty, 1985); *Salmonella sp.* (Black, 1985; Candy *et al.*, 1985; Reynolds *et al.*, 1986; Snodgrass *et al.*, 1986); undifferentiated *E. coli* (Sherwood *et al.*, 1983; Black, 1985; Tzipori, 1985a; Reynolds *et al.*, 1986; Snodgrass *et al.*, 1986; Johnson, 1992); enterotoxigenic *Bacteroides fragilis* (Border *et al.*, 1985; Myers & Shoop, 1987); 'thermophilic' *Campylobacter sp.* (Firehammer & Myers, 1981; Tzipori, 1985b); and *C. perfringens* (Hobbs *et al.*, 1953; Shann *et al.*, 1979; Jewkes *et al.*, 1981; Stringer, 1985; Tzipori, 1985a; Van Kessel *et al.*, 1985; Walker, 1985; Niilo, 1987; Van Baelan & Devriese, 1987; Borriello, 1988; Traub-Dargatz *et al.*, 1988; Collins *et al.*, 1989; Johnson, 1992; Wilkinson, 1992). The relative prevalence rates of different pathogens varies with location and animal species. For example, the major diarrhoeagenic organism in calves is reported as rotavirus (Tzipori, 1985b), whereas in piglets it is enterotoxigenic (ETEC) *E. coli* (Tzipori, 1985b).

Examining a particular pathogen in detail can reveal differences in virulence factors which are important for pathogenesis in different animal species. For example, ETEC have been reported in children (Black, 1985), pigs (Tzipori, 1985b; Johnson, 1992) and calves (Sherwood *et al.*, 1983; Tzipori, 1985a; Reynolds *et al.*, 1986; Snodgrass, 1986; Snodgrass *et al.*, 1986) with a similar pathogenic mechanism, but the fimbrial adhesins involved may be different and host specific. Although an association of ETEC with foals is suspected, none has been found with any known or uncharacterised fimbrial types (Hardy *et al.*, 1991).

4.3.2.3 Treatment and prevention

The loss of water during diarrhoea may result in dehydration which can be treated by oral rehydration aided by sodium, potassium and chloride ions together with glucose in water. Glucose is actively taken up coupled with sodium, and water and chloride ions follow passively. 'Super solutions' contain an additional amino-acid, such as glycine, which is taken up actively and is said to diminish the rate and duration of diarrhoea as well as assisting with rehydration (Levine, 1985). Oral rehydration is a highly effective, and often sufficient treatment.

Probiotics, a dose of bacteria usually present in the healthy gut, are occasionally used to establish or restore a 'normal flora' to the gut to help prevent or to resolve cases of diarrhoea. Antibiotics are occasionally used to treat cases of diarrhoea, but this can lead to an imbalance of the gut flora. Antibiotic treatment itself, may lead to diarrhoea and so is not routinely recommended for the treatment of enteric bacterial infections (George & Finegold, 1985; Buechner, 1989).

A significant reduction in the prevalence of diarrhoea can be achieved with, increased cleanliness at birth, and also by ensuring a good uptake of colostrum, within 6 hours of birth to give passive immunity until the new-born's natural gut immunity matures (Rowley, 1985; Koterba, 1991).

Determining the epidemiology of a disease can help indicate the appropriate preventative measures which should be taken. This may include the development and routine administration of a vaccine. Vaccines can prevent the establishment of organisms in the host either by stimulating antibodies which prevent adhesion, or which are bactericidal or which neutralise toxins. Vaccines are available for a number of enteric pathogens in different species. These include a vaccine for *E. coli* diarrhoea based on different adhesins, live attenuated vaccines for several *Salmonella sp.* and *Vibrio cholerae*, toxoid vaccines of *Clostridium perfringens* and a vaccine against rotavirus (Walker, 1992).

A specific diagnosis helps to target treatments or preventative measures. However, identification of the specific cause of a particular case or outbreak of diarrhoea may be difficult, and often a combination of factors may be involved.

4.3.3 Foal Diarrhoea

Eighty percent of foals may be expected to have one or more episodes of diarrhoea in the first six months of life (Urquhart, 1981). Many cases may be mild and self-limiting, but others can be severe and rapidly lead to death. In a recent survey of foal diarrhoea (Holland *et al.*, 1991), a potential pathogen was not identified in association with 78% of cases. This failure to identify an aetiology may be due to a non-infectious cause, an unrecognised pathogen, or insensitive detection methods for recognised pathogens. It is not always possible to predict which cases of foal diarrhoea will be transient and self limiting or which will be more serious and require intensive therapy before a specific diagnosis can be made. Consequently, there is a need to investigate cases before a systematic management and therapeutic plan can be devised, based on a knowledge of the cause.

However, to enable the determination of an infectious cause of diarrhoea, specific tests are required to detect each potential pathogen from amongst the large numbers of organisms present in the normal flora. Consequently, it is necessary to know the range of organisms that might cause diarrhoea in foals, before a full investigation can be made to give a diagnosis and indication of treatment and control measures.

4.3.3.1 Known and possible causes of Foal Diarrhoea

A syndrome named 'foal heat diarrhoea', is the most commonly diagnosed enteric disease of foals (Whitlock, 1975; Martens, 1979; Urquhart, 1981; Martens & Scrutchfield, 1982; Reed *et al.*, 1983; Palmer, 1985) which occurs between 6 and 14 days of age, lasts 2 to 5 days, is soft to watery in consistency, and is otherwise not profuse with the foal remaining bright. It is usually self-limiting and many possible aetiologies have been proposed. These include hormonal changes during the mare's oestrus period (Martens, 1979; Martens & Scrutchfield, 1982; Palmer, 1985),

changes in milk composition (Martens, 1979; Urquhart, 1981; Palmer, 1985), coprophagy (Martens, 1979; Palmer, 1985), ingestion of genital discharges (Martens, 1979), *Strongyloides westeri* (Whitlock, 1975; Martens, 1979) and physiological changes of the developing intestinal tract (Whitlock, 1975; Martens, 1979; Traub-Dargatz *et al.*, 1988). Although individual cases may have any of these aetiologies, it has been proposed that the majority of cases are likely to be a response to a physiologic change of the intestinal tract (Palmer, 1985) related to changes in intestinal flora or increased fibre consumption (Traub-Dargatz *et al.*, 1988). The transient nature of the diarrhoea may reflect rapid functional maturation of the large intestine (Palmer, 1985). Many of the more serious forms of foal diarrhoea, however, can initially resemble foal heat diarrhoea (Palmer, 1985).

Other reported causes of diarrhoea in foals include overeating, a sudden change in diet or ingestion of foreign fibrous materials (Martens, 1979; Reed *et al.*, 1983) which overwhelms the immature intestine and causes an osmotic influx of water into the gut. Carbohydrate intolerance, a cause of osmotic diarrhoea, may occur as a primary entity or secondary to enteritis (Martens, 1979). Diarrhoea may also develop as a result of antibiotic therapy. The pathogenesis is usually related to: (a) direct toxic effects; (b) alterations in the normal intestinal flora; (c) the development of resistant strains of bacteria; or (d) superinfections by pathogenic organisms. Oral antibiotics, such as the aminoglycosides, which are poorly absorbed from the intestinal tract, are more likely to cause adverse effects in foals (Martens & Scrutchfield, 1982).

Diarrhoea may also occur secondarily to systemic disease, such as, septicaemia, or a physiological condition, such as, gastric impaction, ulceration or neoplasia (Edwards, 1992). Infectious agents can cause some of the more severe cases of foal diarrhoea which can lead to death; these are described below.

Since 'foal heat diarrhoea' and other so-called non-infectious diarrhoea are rather poorly defined and infectious agents have not been thoroughly investigated as part of their potential aetiology they will not be regarded in this

study as separate distinct syndromes and all samples from foal diarrhoea will be investigated microbiologically.

4.3.3.2 Infectious Foal Diarrhoea

Many microbial causes of diarrhoea in foals have been proposed but their prevalence and significance is unclear or variably reported. The most frequently reported bacterial cause is *Salmonella sp.*, with a prevalence ranging from 0 - 78% of foals with diarrhoea (Smith *et al.*, 1978; Carter *et al.*, 1979; Madigan *et al.*, 1990; Browning *et al.*, 1992). *S. typhimurium* (Smith *et al.*, 1978; Browning *et al.*, 1992) is the most commonly identified serotype, but others have been reported such as *S. bovis-morbidificans* (Carter *et al.*, 1979) and *S. ohio* (Madigan *et al.*, 1990). The isolation of 'thermophilic' *Campylobacter sp.* (<1% prevalence; Browning *et al.*, 1991), *Yersinia enterocolitica* (12%; Browning *et al.*, 1991), undifferentiated *Escherichia coli* (100%; Tzipori, 1985b; 22%; Browning *et al.*, 1991) and *Aeromonas hydrophila* (9%; Browning *et al.*, 1991) have all been reported but their significance as a cause of foal diarrhoea remains uncertain.

Other bacteria have been reported as the possible cause of individual cases and outbreaks of diarrhoea and have been the subject of agent specific surveys and studies. These include:

- (1) *Clostridium perfringens* of types A (Dart *et al.*, 1988), B (Montgomerie & Rowlands, 1937; Mason & Robinson, 1938; Stubbings, 1990), C (Dickie *et al.*, 1978; Niilo & Chalmers, 1982; Sims *et al.*, 1985; Howard-Martin *et al.*, 1986; Pearson *et al.*, 1986; Niilo, 1987) and D (Stringer, 1985; Stubbings, 1990), which were reported in association with individual cases as well as outbreaks of foal diarrhoea;
- (2) enterotoxigenic *Bacteroides fragilis*, which was present in 25% of scouring foals (Myers & Shoop, 1987);
- (3) *Clostridium difficile*, present in 63% of scouring foals (Jones *et al.*, 1987) and attributed as the cause in four cases (Jones *et al.*, 1988);

- (4) and *Streptococcus durans*, reported as a possible cause of foal diarrhoea (Tzipori *et al.*, 1984; Tschivdewahn *et al.*, 1991).

Klebsiella pneumoniae (Tzipori, 1985b), *Actinobacillus equuli* (Tzipori, 1985b) and *Rhodococcus equi* (Prescott & Hoffman, 1993) have been occasionally associated with individual cases of foal diarrhoea but these were in association with severe systemic disease of which diarrhoea was probably a subsidiary sign.

Cryptosporidium sp. was reported as occasionally associated with foal diarrhoea (Coleman *et al.*, 1989; Austin *et al.*, 1990; Lyons *et al.*, 1991a) but has been detected in as many as 29% of cases (Browning *et al.*, 1991). *Giardia sp.* (Lyons *et al.*, 1991a) and *Eimeria leukarti* (Lyons *et al.*, 1991a) have also been associated with foal diarrhoea but their prevalence and significance is unknown. An association with diarrhoea has been reported with several helminth species, particularly *Strongyloides westeri* (Lyons *et al.*, 1991b).

Rotavirus has been widely reported as a major cause of foal diarrhoea and its prevalence varied from 8% - 37% of cases (Conner & Darlington, 1980; Strickland *et al.*, 1982; Tzipori, 1985b; Dwyer *et al.*, 1990; Browning *et al.*, 1991; Browning *et al.*, 1992; Holland *et al.*, 1992). Other viruses reported in association with foal diarrhoea are coronavirus (37% prevalence; McNulty, 1985; Tzipori, 1985b) and parvovirus-like particles (Baker & Ames, 1987).

Most information about the significance and prevalence of potential pathogens causing foal diarrhoea has been collected as case reports, single outbreak investigations and specific surveys for individual potential pathogens without extensive differential diagnoses. Surveys that have included a wide range of potential pathogens have, however, concentrated on the study of thoroughbred foals at stud. The statistical analyses of the results of these surveys was limited to calculation of the percentage prevalence of a pathogen in healthy and scouring animals, and determination of a statistically significant difference (univariate) between isolation of an organism from healthy and scouring foals.

4.3.3.3 Justification for a study of the rôle of *Clostridium perfringens* in foal diarrhoea

Although there have been many case reports of suspected pathogens in foal diarrhoea, no comprehensive survey has been undertaken of all potential pathogens in foals, other than of thoroughbreds in a stud environment. There is a need to assess the relative prevalence of potential pathogens concurrently, over several seasons, in several different environments. Prevalence rates may change from one year to another year and between different sub-populations.

A potential pathogen was not identified for between 44% (Dwyer *et al.*, 1990) and 78% (Holland *et al.*, 1991) of cases of foal diarrhoea. Some of these may have had a non-infectious cause, such as overeating or antibiotic treatment (Martens, 1979; Urquhart, 1981; Reed *et al.*, 1983), but it is possible that there are further infectious agents that remain to be identified. This study sets out to assess the significance of *C. perfringens* as one of these potential causes, as well as determining the relative extent and prevalence of several known pathogens.

Salmonella sp. and Rotavirus, which are widely accepted causes of foal diarrhoea, were studied, together with potential pathogens recognised in other animal species, and frequently reported in association with individual cases of foal diarrhoea. These included 'thermophilic' *Campylobacter sp.*, *Yersinia enterocolitica*, *E. coli* (undifferentiated), *Cryptosporidium sp.*, *Strongyloides westeri* and other helminths for whom the significance and prevalence is unclear.

C. perfringens was particularly chosen for in-depth study because of the much larger number of reported associations of *C. perfringens* with individual cases and outbreaks of foal diarrhoea (Montgomerie and Rowlands, 1937; Mason & Robinson, 1938; Dickie *et al.*, 1978; Niilo & Chalmers, 1982; Sims *et al.*, 1985; Howard-Martin *et al.*, 1986; Pearson *et al.*, 1986; Niilo, 1987; Dart *et al.*, 1988; Stubbings, 1990), than reported for other potential pathogens currently recognised in other species and occasionally reported in foals. Although diarrhoea in foals has been investigated in several surveys (Tzipori, 1985b; Dwyer, *et al.*, 1990; Browning *et al.*, 1991; Holland

et al., 1992) *C. perfringens* was either not tested for, or was cultured by methods not specifically designed for isolation from equine faeces. Previous surveys of diarrhoea which did include *C. perfringens* have used one method of isolation, usually with heat shock or alcohol shock, to stimulate endospores to germinate. These methods are often used in studies of human food poisoning where a high count of *C. perfringens* endospores ($>10^6$ cfu (colony forming units) per gram of faeces) is one of the main diagnostic features (Hobbs *et al.*, 1953; Sutton & Hobbs, 1968; Koransky, *et al.*, 1978; Borriello *et al.*, 1985; Brett *et al.*, 1992). However, as these methods are designed to identify the probable presence of a particular pathogenic mechanism of *C. perfringens*, they may be inappropriate for a study of foal diarrhoea if an alternative mechanism of pathogenesis occurs.

4.3.4 Clostridium perfringens as a cause of diarrhoea

Clostridium perfringens in a variety of mammals is associated with a number of different disease syndromes, including diarrhoea. *C. perfringens* can be divided into several types A, B, C, D and E, on the basis of production of four major toxins; alpha (α); beta (β); epsilon (ϵ); and iota (ι). Different pathogenic mechanisms and other virulence determinants (including so-called minor toxins) are associated with each type. Some of the pathogenic mechanisms are associated with the production of some of the minor toxins. Enterotoxin, for example, is a minor toxin associated with diarrhoea. Types A, B, C, D and E have all been implicated in cases of diarrhoea in several animal species (McDonel, 1986). For example, *C. perfringens* type C, which produces α and β major toxins, is associated with necrotic enteritis in humans (Shann *et al.*, 1979; Van Kessel *et al.*, 1985; Walker, 1985; Wilkinson, 1992), calves (Borriello & Carman, 1985; Tzipori, 1985b), pigs (Borriello & Carman, 1985; Tzipori, 1985b), sheep (Borriello & Carman, 1985) and foals (Dickie *et al.*, 1978; Niilo & Chalmers, 1982; Borriello & Carman, 1985; Sims *et al.*, 1985; Tzipori, 1985b; Howard-Martin *et al.*, 1986; Pearson *et al.*, 1986; Niilo, 1987). These 'enterotoxaemic diarrhoeas', are associated with aggressive toxins which establish necrotic lesions in the gut mucosa which are often fatal and typically associated with

severe enteric lesions seen at post mortem examination (Barnes & Moon, 1964; Shann *et al.*, 1979; Borriello & Carmen, 1985; Tzipori, 1985b; Walker, 1985).

Other strains of *C. perfringens* produce a distinct enterotoxin which acts to stimulate fluid hypersecretion from enteric mucosa, and are a cause of food borne outbreaks of diarrhoea in man from which individuals usually recover uneventfully (Stringer, 1985). The same toxin is associated with infectious outbreaks of *C. perfringens* diarrhoea in communal establishments (Borriello *et al.*, 1985). A high rate of enterotoxin production is linked to the formation of endospores and, consequently, food-borne cases were originally detected indirectly by the presence of large numbers of heat resistant *C. perfringens* in faeces (Hobbs *et al.*, 1953). Since then, however, enterotoxin production associated with strains which have heat sensitive endospores has been recognised (Sutton and Hobbs, 1968). In addition, a single sporulation event following ingestion, detected by the presence of large numbers of heat resistant bacteria, may not be a relevant diagnostic feature, as may prove to be the case for *C. perfringens* enterotoxin associated infectious diarrhoea (Borriello *et al.*, 1985; Brett *et al.*, 1992).

4.3.4.1 Enterotoxigenic *Clostridium perfringens*

Enterotoxigenic type A *C. perfringens* is a cause of several distinct syndromes in man. These include food poisoning (Hobbs *et al.*, 1953; Stringer, 1985), antibiotic associated diarrhoea (Borriello *et al.*, 1984; Borriello *et al.*, 1985; Borriello & Larson, 1985; Williams *et al.*, 1985; Samuel *et al.*, 1991), sporadic infectious diarrhoea (Borriello, 1985; Borriello *et al.*, 1987; Borriello, 1988; Larson & Borriello, 1988; Brett *et al.*, 1992), and sudden infant death syndrome (Murrell *et al.*, 1987; Murrell *et al.*, 1993). Enterotoxigenic *C. perfringens* has also been isolated from pigs, cattle, sheep, goats, and horses with diarrhoea (Van Baelan & Devriese, 1987; Traub-Dargatz *et al.*, 1988), but a pathogenic rôle and statistical association with diarrhoea have not been established.

Enterotoxigenic *C. perfringens* has been studied in most depth, in human disease, and although several different pathogenic mechanisms have been considered,

the number of organisms and presence of enterotoxin in the faeces were found to be important in most cases (Hobbs *et al.*, 1953; Borriello *et al.*, 1984; Borriello, 1985; Borriello & Larson, 1985; Borriello *et al.*, 1985; Stringer, 1985; Williams *et al.*, 1985; Borriello *et al.*, 1987; Murrell *et al.*, 1987; Borriello, 1988; Larson & Borriello, 1988; Samuel *et al.*, 1991; Brett *et al.*, 1992; Murrell *et al.*, 1993). Enterotoxin acts by changing the permeability of the plasma membrane of mammalian cells. It first binds to a protein receptor on the membrane, gradually becoming more resistant to removal by proteases, and forms a large complex consisting of one *C. perfringens* enterotoxin molecule, one 70 kDa membrane protein, and one 50 kDa membrane protein. The plasma membrane then becomes freely permeable to small molecules such as ions and amino acids, leading to secondary cellular effects and cell death. Loss of intestinal epithelial cells probably causes, or strongly contributes to, intestinal fluid loss (which is manifest as diarrhoea).

Enterotoxin is produced in large amounts when enterotoxigenic strains of *C. perfringens* sporulate. Toxin is released along with the endospore when the vegetative cell lyses. Thus, the presence of enterotoxin and a count of 10^6 endospores per gram faeces is considered diagnostic for food poisoning. However, the same association between sporadic infectious diarrhoea and large numbers of endospores in individual cases is not necessarily seen (Brett *et al.*, 1992). Large numbers of *C. perfringens* organisms are ingested with food, in food poisoning cases, followed by a sporulation event which results in large numbers of *C. perfringens* endospores and enterotoxin which are shed in the faeces. In contrast with sporadic infectious diarrhoea, there is, perhaps, a gradual build up of vegetative cells, leading to successive sporulation events and enterotoxin production, which need not, therefore, produce a peak of very large numbers of endospores in faeces.

C. perfringens types B, C, and D disease usually include a severe enteritis, often necrotic and fatal, whereas enterotoxigenic type A *C. perfringens* infections are often milder and more likely to be confused with those clinical signs produced by other diarrhoeagenic organisms. Thus the isolation and enumeration of

enterotoxigenic and non-enterotoxigenic *C. perfringens* might indicate both the strength of association with diarrhoea as well as which mechanism, whether 'food poisoning-like' or 'infectious diarrhoea-like', is operating.

4.4 OBJECTIVES

The objectives of the study were:

1. To establish sensitive methods for *C. perfringens* (a) selective isolation; (b) overall viable count; and (c) endospore count.
2. To identify and set up appropriate methods for the isolation and identification of each of the recognised pathogens in foal diarrhoea.
3. To use these methods in a case / control survey of a variety of breeds of foals, at stud and other types of premises, throughout the United Kingdom.
4. To determine the relative prevalence of:
 - *Salmonella* sp.
 - Rotavirus
 - 'thermophilic' *Campylobacter* sp.
 - *Yersinia enterocolitica*
 - *Escherichia coli*
 - *Cryptosporidium* sp.
 - *Strongyloides westeri* and other helminths, and
 - *Clostridium perfringens*.
5. To determine which of these organisms is significantly associated with diarrhoea by multivariate logistic regression analysis.
6. To determine the association between diarrhoea and enterotoxigenic *C. perfringens*.
7. To determine the sequence of the *C. perfringens* enterotoxin gene from an equine isolate and compare it with the sequence previously published for the gene from a human isolate to establish its identity at the molecular level.

8. To determine the association of *C. perfringens* with the enterotoxin gene with foal diarrhoea by polymerase chain reaction and hybridisation of chromosomal DNA with an enterotoxin gene probe.*

* Footnote- These objectives will be addressed in the following chapters: Objective 1 - see Chapter Two; Objectives 2 to 5 - see Chapter Three; Objectives 6 & 7 - see Chapter Four; Objective 8 - see Chapter Five.

5.0 CHAPTER TWO

INCREASING THE SENSITIVITY OF *CLOSTRIDIUM PERFRINGENS* ISOLATION FROM FOAL FAECES

5.1 CONTENTS

5.1.1 List of Tables	34
5.1.2 List of Figures	35
5.2 Introduction	36
5.2.1 The study of foal diarrhoea and <i>C. perfringens</i>	36
5.2.2 The isolation and identification of <i>Clostridium perfringens</i>	36
5.2.2.1 Cultural and biochemical characteristics of <i>C. perfringens</i>	36
5.2.2.2 Culture of <i>C. perfringens</i> endospores	41
5.3 Objectives	46
5.4 Methods and materials	47
5.4.1 Bacteria	47
5.4.2 Media and culture conditions	47
5.4.3 Samples of foal faeces	48
5.4.4 Pre-treatment of cultures or faecal suspensions	48
5.4.5 Identification of <i>C. perfringens</i>	48
5.4.6 Enumeration of endospores and viable <i>C. perfringens</i>	48
5.5 Results and Discussion	50
5.5.1 The effect of different pre-treatments and culture conditions on the recovery of endospores	50
5.5.2 Isolation of <i>C. perfringens</i> from foal faeces by different methods	51

5.1.1 List of Tables

Table 1. Recovery of <i>Clostridium perfringens</i> NCTC8239 from a sporulated culture of different ages treated at 80°C for different times (% of total endospore count before treatment)	53
Table 2. Recovery of <i>Clostridium perfringens</i> NCTC8239 from a sporulated culture of different ages treated at 60°C for different times (% of total endospore count before treatment)	53

Table 3. Recovery of *Clostridium perfringens* NCTC8239 from a sporulated culture of different ages treated with 50% ethanol at 25°C for different times (% of total endospore count before treatment). 54

Table 4. Mean recovery of *Clostridium perfringens* NCTC8239 from 22 hour sporulated cultures by different endospore stimulation methods (percentage of total endospore count before treatment)..... 54

Table 5. Isolation of *Clostridium perfringens* from 271 equine faeces by five different methods. 55

5.1.2 List of Figures

FIGURE 1. Distribution of the number of *C. perfringens* isolations from foal faeces by direct isolation methods (n = 271)..... 55

FIGURE 2. Distribution of the number of *C. perfringens* isolations from foal faeces samples by enrichment methods (n = 271) 55

FIGURE 3. Comparison of distribution of the number of *C. perfringens* isolations from foal faeces samples by direct culture and enrichment methods (n = 271) 55

5.2 INTRODUCTION

5.2.1 The study of foal diarrhoea and *C. perfringens*

An evaluation of the rôle of *C. perfringens* in foal diarrhoea without assumptions about pathogenesis in relation to sporulation would require the detection of heat sensitive forms, including some endospores, as well as heat resistant endospores and the identification of sporulation events that might be epidemiologically important. Additionally, variation in optimal conditions for endospore germination by different strains of *C. perfringens* might have to be taken into account for sensitive isolation of as many strains of these bacteria as possible.

5.2.2 The isolation and identification of *Clostridium perfringens*

C. perfringens is widely spread in the environment. Type A strains (see below for definition of the different types) have been found in almost every sample of soil and almost every species of animal that has been tested. Types B, C, D and E are usually restricted to the intestinal tract, primarily of mammals, and including occasionally of man. Types B, C and D have been isolated from soil in areas where enteritis was affecting significant numbers of animals or humans, but they appear to be unable to compete with the better-adapted normal soil inhabitants (Smith & Williams, 1984).

5.2.2.1 Cultural and biochemical characteristics of *C. perfringens*

C. perfringens is a Gram-positive rod, approximately 4 - 8 by 0.8 - 1.5 micrometres, with parallel sides and rounded ends. *C. perfringens* is one of the most rapidly growing anaerobes; even in the presence of small amounts of oxygen. Surface growth is often detectable after only four to six hours incubation. Surface colonies are circular, about 2 - 4 mm in diameter after 24 hours incubation, convex, semi-translucent, smooth and with an entire edge. Less commonly, colonies are umbonate, with radial striations and have a crenated or scalloped edge (Bergey, 1994). Many strains of *C. perfringens* produce zones of complete Beta-haemolysis on horse blood agar due to the production of theta toxin, and partial haemolysis is frequently seen among type A strains, due to the action of alpha toxin. Those strains producing theta

as well as alpha toxin will show two zones of haemolysis, a narrow band of complete haemolysis and a wider zone of partial haemolysis (Evans, 1945). After exposure to low concentrations of alpha toxin at 37°C, erythrocytes of many species do not undergo haemolysis until cooled below a critical temperature. This so called 'hot-cold lysis' is seen commonly with sheep erythrocytes but less so with horse erythrocytes, and very little with those of the rabbit or man (Meduski & Hochstein, 1972).

Specimens to be examined for *C. perfringens* should not be refrigerated as this organism dies at low temperatures and false negative results may be obtained (Smith & Williams, 1984). However, storage at lower temperatures, minus 70°C for example, does not result in the same loss of numbers.

The optimal temperature for rapid growth of strains of Type A, D and E is about 45°C, but production of toxins is best at lower temperatures. Mean generation times can be extremely short, sometimes as little as 8 minutes. Strains of types B and C usually grow at about the same rate at both 37°C and 45°C (Smith & Williams, 1984). It has been observed that when the organism is grown at 50°C, most of the cells in the inoculum die within a few hours but thereafter, the survivors multiply rapidly: this is called the Phoenix Phenomenon. It involves a process of heat injury / cold-shock injury which occurs during the counting and recovery of bacterial cells when they are transferred from growth medium at 50°C to diluent at room temperature. Chemical injury, brought about by oxygen and oxidized compounds in the medium, is also involved. Injury can be avoided by using strictly anaerobic media equilibrated at the incubation temperature (Shoemaker & Pierson, 1976). The pH range at which growth takes place is from 5.5 to 8.0, but the optimal value for rapid growth is pH 6.5. The growth of *C. perfringens* is affected by salt concentrations of 3% or higher (Smith & Williams, 1984).

The short generation time of *C. perfringens*, its lack of susceptibility to low concentrations of oxygen and its relative resistance to such compounds as sodium sulphite, sulphadiazine, polymyxin, neomycin, kanamycin, and cycloserine, have together allowed the design of several media for its isolation or enumeration. Many

selective media devised for isolating *C. perfringens* contain sulphite and some iron salt. Clostridia are resistant to sulphite, whilst other bacteria are inhibited. Most clostridia reduce sulphite to sulphide which, in media containing iron, will give rise to black colonies or black zones around colonies. The minimal nutrient requirements to be added to the medium for this organism include eleven amino-acids and two vitamins (Sebald & Costilow, 1975). The most common solid media for presumptive enumeration of *C. perfringens* include SPS (sulphite polymyxin sulphadiazine), TSN (tryptose sulphite neomycin), SFP (Shahidi- Ferguson perfringens), TSC (tryptose sulphite cycloserine), and OPSP (oleandomycin polymyxin sulphadiazine perfringens). Most of the titles for these media indicate the main selective and differential constituents, except SFP which contains kanamycin sulphate and polymyxin B sulphate as selective agents. In addition to all the basic nutrients required for growth, they also contain an iron salt and sulphite so that *C. perfringens* and other sulphite reducing agents such as *Salmonella sp.*, *Proteus sp.*, *C. bifermentans*, and *C. butyricum* may produce black colonies.

The aim of selective media is to inhibit the growth of other sulphite reducing bacteria allowing *C. perfringens* to grow and therefore to be selected and differentiated from other non-sulphite / sulphate reducing bacteria. Some faecal streptococci may grow on clostridial media but these will remain small white colonies, easily distinguishable from the large black colonies of *C. perfringens*. *C. bifermentans* and *C. butyricum* will grow on SFP and TSN media as black colonies with a tendency to spread and obscure the whole surface of the agar. The less stringent selective agents of SFP agar allows a greater number of *C. perfringens* organisms to be isolated than SPS or TSN agar, but a greater number of non-clostridial colonies were found on SFP agar (Smith & Williams, 1984; Harman & Kaulter, 1987). Egg yolk is included in SFP agar and is optional in TSC agar. Most strains of *C. perfringens* may produce an opaque zone around the colony in media containing egg yolk due to lecithinase activity, but this is not a universal characteristic since occasional strains may not produce alpha toxin or sufficient alpha toxin to

detect in this way. Indeed, both black lecithinase positive and black lecithinase negative colonies should be considered as presumptive *C. perfringens* on TSC or SFP agars and confirmatory tests should be carried out (Bridson, 1990).

Carbohydrates fermented by *C. perfringens* include fructose, glucose, galactose, inositol, lactose, maltose, mannose, starch and sucrose. A few strains will ferment cellobiose, glycerol, inulin, raffinose or salicin. Fermentation products include acetic and butyric acids, sometimes with butanol. Rapid fermentation of the lactose in ordinary milk medium occurs with the subsequent development of a stormy clot reaction. Gelatin is hydrolysed and a few strains hydrolyse casein. Urease is produced by some strains and acid phosphatase by almost all strains. On egg-yolk agar, *C. perfringens* alpha toxin produces diffuse opalescence which is inhibited by *C. perfringens* Type A anti-toxin.

A number of strains of *C. perfringens* have capsules which are enhanced if grown at a pH slightly above neutrality in the presence of a fermentable carbohydrate and serum or milk. The capsule which can be seen by negative staining with india ink is composed of complex polysaccharides loosely combined with peptides.

Different isolates of *C. perfringens* produce at least twelve different soluble substances which are loosely referred to as toxins. Four of the toxins which are each lethal for mice are called alpha, beta, epsilon and iota (Niilo, 1980). These are the 'major' toxins the different patterns of possession of which are used to group the species into five toxigenic types: A, B, C, D and E. Type A strains produce alpha toxin; type B strains produce alpha, beta and epsilon toxins; type C strains produce alpha and beta; type D strains produce alpha and epsilon toxins; and type E strains produce alpha and iota toxins (Oakley & Warrack, 1953; McDonel, 1986). A strain originally proposed as type F (Zeissler & Rassfeld-Sternberg, 1949), has since been more accurately classified as a sub-type of type C (Sterne & Warrack, 1964). Typing antisera are traditionally used to neutralise the lethality of crude culture supernatants for mice. Clearcut distinctions in this assay do not always exist between different types of *C. perfringens*, and erroneous typing results can arise (Serrano & Schneider,

1978). Variants which do not produce particular toxins are found, and degraded types are those which originally possessed type toxins but subsequently lost their ability to produce a certain toxin. A wide variation exists in amounts of toxin produced by different strains, which affects the ability of antisera to sufficiently neutralise the activity of some strains when using typing methods which involve toxin detection or neutralisation tests in mice.

C. perfringens type A can be sub-divided by means of the polysaccharide antigens in the capsular layer. This typing system was originally developed for heat resistant strains (Hobbs *et al.*, 1953) but now includes heat sensitive strains also (Stringer *et al.*, 1980). Other typing methods which have been tried, include bacteriophage, bacteriocin, plasmid and esterase electrophoretic typing (Yan, 1989; Mahony *et al.*, 1992; Pons *et al.*, 1993).

One of the more important 'minor' toxins, enterotoxin, is formed mostly during sporulation, although it has been demonstrated that it can be produced in small amounts in cultures that have not sporulated (Granum *et al.*, 1984; Goldner *et al.*, 1986). Enterotoxin is most commonly formed by type A strains of *C. perfringens* but can be produced by some isolates of types C and D (Skelkvale *et al.*, 1979). Some strains of type A *C. perfringens* produce enterotoxin and can cause diarrhoea in man and other animal species, such as pigs and calves (Tzipori, 1985b; Van Baelan & Devriese, 1987; Collins *et al.*, 1989). As enterotoxin production is promoted during sporulation, a correlation between the presence of enterotoxin and large numbers of endospores is often observed in certain syndromes, such as in human food poisoning.

Endospores are thought to form when the organisms' surrounding environment becomes adverse so as to act, as a protective mechanism. The spores are large, oval and central or subterminal, and distend the organism. The spore coat is composed of at least four proteins that, together with the enterotoxin, are formed early in the process of sporulation (Labbe & Duncan, 1977). Endospores are rarely seen in artificial culture conditions used for isolation and their absence in a culture of clostridia is one of the characteristic features of *C. perfringens*. Sporulation is said to

be favoured by an alkaline environment and by the absence of carbohydrates fermentable by *C. perfringens*. Media designed to encourage *C. perfringens* to sporulate include: a magnesium sulphate peptone medium (Ellner, 1956), a peptone starch medium (Duncan & Strong, 1968), and a glucose ion-exchange resin medium (Clifford & Anellis, 1971). These media allow some active growth of *C. perfringens*, followed by sporulation when the small amount of carbohydrate present is exhausted.

The process of sporulation involves seven stages. At stage I the cell has stopped growing and the compact nucleus has spread out and formed a filament. At stage II an asymmetrical cell division occurs, with the two segments of protoplasm being separated by asymmetrical cell division each containing half of the DNA of the original cell. Starting from the asymmetrical septum, the smaller segment of protoplasm becomes engulfed by the membrane of the larger. At this stage the cell is committed to sporulate or the cell will be unable to survive. Between the membranes of the smaller and larger protoplasts, a peptidoglycan cortex is laid down during stage III to IV. During stages V and VI the protein endospore coat is assembled over the outer surface membrane which was originally part of the larger protoplast and in the last stages heat resistance develops. At stage VII the endospore is released from the mother cell.

5.2.2.2 Culture of *C. perfringens* endospores

Clostridium perfringens endospores require pretreatment, for *in vitro* culture, before they will germinate; this process is called activation. The presence and number of *C. perfringens* endospores in a faecal sample, can be assessed by using the following techniques: heat activation at 70°C - 80°C, for 20 minutes (Tsai & Riemann, 1974) or alcohol treatment (50% ethanol, 1 hour; Koransky *et al.*, 1978). However, when these techniques are used on a sample of endospores of a known concentration, full recovery of a colony forming unit per endospore seen microscopically is not usually achieved. This may be due to a number of reasons including non-activation, injury of activated or germinating endospores or a failure of germination.

Nutritive stimulus alone is often not sufficient to achieve full endospore germination. Activation methods are, therefore, used to enhance the germination process.

Activation of bacterial endospores is known to change the qualitative and quantitative requirements for the induction of germination, to decrease germination lag (Hashimoto *et al.*, 1972), to increase the overall rate of germination (Keynan & Evenchik, 1969), to increase the rate of commitment to germinate (Stewart *et al.*, 1981), and to activate certain enzymes in resting endospores (Ando, 1979; Craven, 1988). The heat or chemical activation of endospores (Levinson & Feeherry, 1978), would denature or cause conformational changes in proteins. This is thought to be the primary trigger in activation, occurring possibly in the endospore coat region (Kenyan *et al.*, 1964; Srivastava & Fitz-James, 1981), or endospore membrane (Keynan, 1978; Vary, 1978). These changes could uncover the active enzymatic sites necessary for germination or facilitate access to binding sites of germinants (agents, such as lysozyme, which facilitate germination; Kenyan *et al.*, 1964).

The germinant receptor site for triggering endospore germination is thought to be hydrophobic in character (Yasuda & Tochikubo, 1984) and the kinetics of activation of *C. perfringens* endospores by alcohols suggests that activation occurs at a hydrophobic site (Craven & Blankenship, 1985). Measurable changes in hydrophobic characteristics of the endospores were also detected after heat activation (Craven & Blankenship, 1987).

The treatments that enhance the activation of endospores of *C. perfringens* NCTC8679 include increased temperature (Tsai & Riemann, 1974), higher pH (Ando, 1978), the presence of chaotropic ions (Craven, 1988), alcohols (Koransky *et al.*, 1978; Craven & Blankenship, 1985), anaesthetics (Craven, 1988) or urea (Craven, 1988). All of these treatments are known to disrupt or weaken hydrophobic interactions. Exposing endospores to conditions that modify hydrophobic interactions of biological macromolecules perturbs the activation site and demonstrates the contribution of hydrophobic forces in maintaining dormancy. Treatments which

retard *C. perfringens* NCTC8679 endospore activation include a decrease in temperature, decrease in pH and a presence of antichaotropic ions which strengthen hydrophobic attractions. It has been suggested that activation involves a conformational change of an endospore protein through weakening of hydrophobic molecular forces and that activation and injury occur at different endospore sites (Craven, 1988).

Following activation, an endospore lytic enzyme is stimulated which degrades the cortex-peptidoglycan. This leads to germination, as judged by the losses of absorbency or refractivity, and heat resistance (Adams, 1978; Ando, 1979; Ando & Tsuzuki, 1984; Ando *et al.*, 1985). Germination is completed with vegetative outgrowth and is characterised by the sequential synthesis of RNA, protein, cell membrane, cell wall and DNA (Adams, 1978).

If conditions employed for the activation of *C. perfringens* endospores are too extreme, they may also cause inactivation of the germination mechanism. Endospores would then be classified as injured; such injury can be avoided through the use of non-nutritive germination agents such as lysozyme (Adams, 1978). Lysozyme attack of the endospore cortex results in germination of those endospores which are sensitive to lysozyme. Some endospores are naturally sensitive to the action of lysozyme, but others first require a pretreatment such as with heat.

Heat injury, in addition to inactivating the normal enzymic germination system, appears also to alter the permeability barrier allowing access of other germinating agents such as lysozyme. The direct access of lysozyme to its substrate can be enhanced, with or without pre-existing heat injury, by alkali or EDTA treatment. Thus to recover the maximum number of *C. perfringens* colony forming units, most endospores must first be sensitised to lysozyme with, for example, EDTA, or sodium hydroxide (Adams, 1973a; Adams, 1973b; Adams, 1974; Adams, 1978). The mechanism of EDTA sensitisation is not known but the greater activity at a higher pH suggests that its rôle is more than to remove lysozyme inhibiting cations (Chang & Carr, 1971) from the endospore surface.

Injury may be reflected in a requirement for a lower optimum temperature for the growth and enumeration of viable cells, due to the activation of a hypothetical alternative germination mechanism. Injury may also increase sensitivity to inhibitors, such as antibiotics, sodium chloride, nitrate and nitrite, because of damage to the endospore plasma or cortical membranes which may be repaired during outgrowth; injury may alter nutritional requirements or other factors such as electric potential (Adams, 1978).

Consequently, when counting 'viable' *C. perfringens* endospores, many factors may need to be taken into account to recover the maximum number of colony forming units. A further complication is that the original population of endospores may not be homogenous in their response to activation agents. For example, the biphasic survivor curves observed for endospores from thermophilic anaerobes at lower thermal inactivation temperatures can be explained in terms of a heterogeneous population of endospores with differing heat stability.

Extreme thermal resistance has correlated with the ratio of endospore cortex volume to endospore cytoplasmic volume (Hyun *et al.*, 1983). Some endospores have been observed by electron microscopy in mixed populations, in which the cortex was either lacking or incomplete. Thus, the heterogeneous population of abortive and mature endospores would result in biphasic survivor curves at a low temperature. At a high temperature, however, rapid inactivation of incompletely formed endospores during the heating process would result in only mature endospores surviving and growing and, hence, linear survivor curves.

Germination sites may also vary in a population. For example, a fraction of the population may rely on a heat sensitive germination system, whilst the rest rely on a multiple site germination system. This variation would provide for a longer resistance to heat treatment whilst endospores with a heat sensitive germination would be inactivated (Ababouch *et al.*, 1987).

Study of the response to specified treatments relies on artificially producing endospores of an homogeneous population. However, in a clinical specimen it is

likely that the population of endospores will be heterogeneous, and so several recovery methods may be required to achieve a true assessment of the number of endospores present or the number of specimens containing *C. perfringens*.

5.3 OBJECTIVES

The objectives of the experiments in this chapter were:

1. To compare the effects of different conditions, encompassing those used traditionally, on the recovery of strains producing heat sensitive and heat resistant endospores.
2. To identify different culture conditions based on these comparisons which would increase the sensitivity of *C. perfringens* isolation from foal faeces.
3. To compare the isolation of *C. perfringens* from foal faeces by methods designed to favour the growth of bacteria in different states of development.

5.4 METHODS AND MATERIALS

5.4.1 Bacteria

C. perfringens NCTC8239, which produces heat resistant endospores, and *C. perfringens* NCTC8237, which produces heat sensitive endospores, were obtained from the National Collection of Type Cultures, Colindale, London.

5.4.2 Media and culture conditions

Robertsons cooked meat medium (cooked meat medium; Oxoid, Unipath) was used for pre-enrichment of faeces; 4.5 ml broths were inoculated with 0.5 ml of a 10% (w or v / v) suspension of faeces in distilled water and incubated at 37°C for 18 hours in 10% carbon dioxide (v / v), 10% (v / v) hydrogen and 80% (v / v) nitrogen. Tryptose Sulphite Cycloserine agar (TSC; Oxoid, Unipath), supplemented where indicated with 1 µg / ml (w / v) lysozyme (Adams, 1973b; supplied by Sigma), was used for spread plate inoculation of 0.1 ml of serial dilutions of faeces or pretreated cultures in distilled water to enumerate clostridia, or streak plate inoculation of pre-enrichment broths or faeces to detect their presence from amongst other bacteria able to grow on the medium used. Plates were incubated at 37°C initially for 18 hours in 10% carbon dioxide (v / v), 10% (v / v) hydrogen and 80% (v / v) nitrogen and subsequently for up to 4 days with daily inspection. TSC agar was used because it not only offers the advantage of selection and differentiation but is also as equally sensitive as Wilkins Chalgren Blood Agar (Oxoid, Unipath) for *C. perfringens* isolation (unpublished observation).

Duncan and Strong medium (Duncan and Strong, 1968) was used to produce endospore containing cultures; 20 ml of medium was inoculated with 2ml of a culture in cooked meat medium and incubated at 37°C for up to 166 hours in 10% (v / v) carbon dioxide, 10% (v / v) hydrogen and 80% (v / v) nitrogen.

Vegetative bacterial cell cultures were produced by inoculation and incubation of Wilkins Chalgren Blood agar (Oxoid) and resuspension of endospore free growth in Fastidious Anaerobe Broth (LabM) or sterile distilled water. Smears were examined microscopically to ensure that no endospores were present.

5.4.3 Samples of foal faeces

Samples of faeces from foals less than 6 months of age were collected from local stud farms and cultured the day they were collected. Occasional samples were sent by post from stud farms further away and these were cultured on the day of arrival. Samples were not refrigerated because of the sensitivity of *C. perfringens* to temperatures just above 0°C (Smith & Williams, 1984).

5.4.4 Pre-treatment of cultures or faecal suspensions

Five millilitre volumes of 10% (w / v) faeces or diluted cultures were made in distilled water previously equilibrated at 60°, 70° or 80°C before incubation in water baths at the same temperature for different times. Where specified, ethanol was added prior to heat treatment to 10% (v / v), or alternatively was used without heat at 50% (v / v).

After heat treatment ethylenediaminetetraacetic acid (EDTA; Sigma) was added to 20 mM, where specified, and the suspension was incubated at 45°C for 1 hour (Adams, 1973b) before sub-inoculation onto TSC medium.

5.4.5 Identification of *C. perfringens*

C. perfringens was presumptively identified on TSC by its typical colony morphology, its black colouration and usual surrounding zone of opalescence. A presumptively identified isolate from each sample for each method was further characterised by its pattern of fermentation of lactose, sucrose, glucose, (acid produced) and salicin (acid not produced) and production of gelatinase and lecithinase (Cowan, 1974).

5.4.6 Enumeration of endospores and viable *C. perfringens*

Colony forming units (cfu) / ml were determined by surface viable count (Cruickshank, *et al.*, 1975). Serial 10-fold dilutions (0.1 ml) of pre-treated or untreated cultures or faeces samples in 0.1% (w / v) peptone water (Oxoid) were spread inoculated onto TSC medium which was incubated at 37°C initially for 18 hours in 10% (v / v) carbon dioxide, 10% (v / v) hydrogen and 80% (v / v) nitrogen and subsequently for up to 4 days with daily inspection. The number of colony

forming units / ml were calculated from the number of colonies growing and the dilution giving 30 - 300 colonies per plate.

Total numbers of vegetative cells and endospores were determined by dark ground microscopic examination of suspensions in an improved Neubauer counting chamber (Cruickshank, *et al.*, 1975), and by counting the number of cells and endospores in a suspension of known volume and dilution. The endospores are highly refractile and can be easily differentiated from vegetative cells.

5.5 RESULTS AND DISCUSSION

5.5.1 The effect of different pre-treatments and culture conditions on the recovery of endospores.

Initial experiments compared the effects of pre-treatment at 80°C, 60°C or exposure to 50% (v / v) ethanol for different times on cultures in Duncan and Strong medium of different ages (Tables 1 - 3). The percentage of endospores (expressed as a fraction of the total count) increased markedly with culture age and hence older cultures contained a proportion of more mature endospores. Generally, there were no significant differences between treatments but the greatest rates of recovery following heating at 80°C seemed to occur after treatment for between 5 and 20 minutes (mean recovery of less than or equal to 20 minutes treatment was greater than the mean recovery of greater than 20 minutes treatment) with a trend of increased recovery with greater culture age (Table 1; see cultures of greater than or equal to 70 hours after 5 to 20 minutes of treatment). In contrast, following heating at 60°C the greatest rates of recovery occurred at 180 minutes of treatment but the largest proportion of endospores were stimulated in younger cultures of less than 94 hours old (Table 2).

After alcohol treatment, recovery of younger cultures of *C. perfringens* was greater than from older cultures and was unaffected by the length of treatment (Table 3); conversely older cultures (70 to 166 hours) were better recovered after a longer exposure (greater than 60 minutes) to ethanol.

Nonetheless, a striking feature of these observations was that only a small proportion of the endospores present could be stimulated to germinate and grow, or otherwise could be recovered, irrespective of the treatment used. Recovery following exposure to 50% (v / v) ethanol overall was less than that seen with either of the heat treatments.

The relatively poor rates of recovery following ethanol treatment prompted a comparison of combined heating and ethanol treatment, with heat treatment alone but at the much lower concentration of ethanol (10% v / v). The use of an additional

recovery period in EDTA after heat / ethanol treatment but before inoculation of growth medium was also assessed. Very similar results were obtained with two trials of this comparison; the means of these results are presented in Table 4. Ethanol treatment combined with heating was still not as effective as heat alone (Table 4: compare treatments at 70°C) but when an EDTA recovery period was also used this trend was reversed and resulted in the highest rates of endospore recovery that were seen. Endospores of strain NCTC8237, which are known to be heat sensitive were not recovered by any of these methods.

5.5.2 Isolation of *C. perfringens* from foal faeces by different methods

Comparison of the effects of different pre-treatments on the recovery of heat sensitive and resistant strains of *C. perfringens* indicated that a wide variety of conditions would be needed to improve the isolation of these bacteria in clinical specimens. To test this hypothesis five culture conditions were simultaneously applied to 271 samples of foal faeces. These methods included pre-enrichment in cooked meat, pre-heating with or without alcohol, pre-treatment with heat before pre-enrichment and direct culture as detailed in Table 5. Sixty-four per cent of the samples were positive for *C. perfringens* by at least one method. However, the isolation rate of any one method was less than that of these methods combined (Table 5).

The most sensitive individual method was pre-enrichment followed by subculture on agar, but this detected only 74% of the positives detected by all methods together. All of the methods isolated *C. perfringens* in at least one sample where the other methods failed (Table 5 & Figures 1, 2 & 3) and in 17% of positives at least one of the pre-enrichment methods failed where a direct culture method succeeded in growing these bacteria.

Comparison of direct isolation methods, with or without pretreatments revealed that quite large proportions of isolations were by one method alone (Figure 1). A similar picture prevailed when comparing different enrichment methods (Figure 2) as well as direct with enrichment methods (Figure 3). These results

confirm a wide distribution of *C. perfringens* in different physiological and development states in foal faeces and that several methods of isolation would be required to assess these bacteria in a microbiological survey of foal diarrhoea.

Table 1 Recovery of *Clostridium perfringens* NCTC 8239 from a sporulated culture of different ages treated at 80°C for different times (% of total endospore count before treatment).

Culture age (hours)	Before treatment			5 minutes	10 minutes	20 minutes	30 minutes	60 minutes	180 minutes
	Viable count (cfu/ml)	Total count	% of total count as endospores						
22	5.8x10 ⁶	1.9x10 ⁹	20.7	1.9	1.7	1.8	1.6	1.6	1.6
46	9.7x10 ⁵	8.5x10 ⁸	26.5	2.3	2.6	2.8	2.6	2.8	2.8
70	3.6x10 ⁶	4.2x10 ⁸	46.5	3.1	3.2	3.4	2.9	3.4	1.9
94	9.6x10 ⁵	3.0x10 ⁸	77.0	2.0	3.3	2.6	2.9	2.3	2.4
166	2.8x10 ⁶	2.8x10 ⁸	82.1	3.0	4.6	2.5	2.8	2.3	1.8

Vegetative cells of *Clostridium perfringens* NCTC8239 grown on blood agar and resuspended in fastidious anaerobe broth did not contain endospores and was killed by treatment at 80°C for 5 minutes.

Table 2 Recovery of *Clostridium perfringens* NCTC 8239 from a sporulated culture of different ages treated at 60°C for different times (% of total endospore count before treatment).

Culture age (hours)	Before treatment			5 minutes	10 minutes	20 minutes	30 minutes	60 minutes	180 minutes
	Viable count (cfu/ml)	Total count	% of total count as endospores						
22	5.8×10^6	1.9×10^9	20.7	1.2	1.2	1.1	1.0	1.3	2.2
46	9.7×10^5	8.5×10^8	26.5	0.7	1.2	1.6	1.9	1.8	2.2
70	3.6×10^6	4.2×10^8	46.5	1.1	1.0	1.3	1.0	1.1	2.6
94	9.6×10^5	3.0×10^8	77.0	0.8	0.8	1.3	0.7	0.8	1.3
166	2.8×10^6	2.8×10^8	82.1	0.4	0.3	0.3	0.4	0.4	1.6

50%, 0.004% and 0% of vegetative cells of *Clostridium perfringens* NCTC8239 grown on blood agar and resuspended in fastidious anaerobe broth (without endospores) survived treatment at 60°C for 5, 10 and 20 minutes, respectively.

Table 3 Recovery of *Clostridium perfringens* NCTC 8239 from a sporulated culture of different ages treated with 50% ethanol at 25°C for different times (% of total endospore count before treatment).

Culture age (hours)	Before treatment			5 minutes	10 minutes	20 minutes	30 minutes	60 minutes	180 minutes
	Viable count (cfu/ml)	Total count	% of total count as endospores						
22	5.8x10 ⁶	1.9x10 ⁹	20.7	1.9	1.1	1.2	1.4	1.5	1.3
46	9.7x10 ⁵	8.5x10 ⁸	26.5	0.6	0.8	0.8	0.6	0.8	0.7
70	3.6x10 ⁶	4.2x10 ⁸	46.5	0.7	0.7	0.7	0.7	0.6	1.1
94	9.6x10 ⁵	3.0x10 ⁸	77.0	0.4	0.5	0.4	0.4	0.4	0.7
166	2.8x10 ⁶	2.8x10 ⁸	82.1	0.3	0.3	0.4	0.4	0.5	0.8
Vegetative cells of <i>Clostridium perfringens</i> NCTC8239 grown on blood agar and resuspended in fastidious anaerobe broth did not contain endospores and was killed by treatment with 50% ethanol at 25°C for 5 minutes.									

Table 4 Mean recovery of *Clostridium perfringens* NCTC8239 from 22 hour sporulated cultures by different endospore stimulation methods (percentage of total endospore count before treatment).

Treatment with 20 mM EDTA (1 hour, 45°C) after heat / ethanol	Pre-treatment before culture on agar medium			
	70°C for 20 minutes in distilled water	50°C for 10 minutes in 10% ethanol	60°C for 10 minutes in 10% ethanol	70°C for 10 minutes in 10% ethanol
+	17.8	1.8	5.7	43.0
-	9.3	0.6	4.2	5.6
A culture of <i>Clostridium perfringens</i> NCTC8237 containing heat sensitive endospores was killed by all of these treatments.				

Table 5 Isolation of <i>Clostridium perfringens</i> from 271 ^a equine faeces by five different methods.			
Isolation method	Number positive	Percentage positive	Percentage positive by method only (number)
Direct culture on agar ^b (Direct plate culture)	72	27	1.8 (5)
Pre-treatment at 70°C for 20 minutes and direct culture on agar ^d (Heat treatment)	72	27	0.7 (2)
Pre-treatment at 70°C for 10 minutes in 10% ethanol and direct culture on agar ^d (Alcohol treatment)	72	27	0.5 (1)
Pre-treatment at 70°C for 20 minutes and Pre-enrichment in broth ^c subculture on agar ^d (Pre-heated enrichment)	116	43	5.5 (15)
Pre-enrichment in broth ^c and subculture on agar ^b (Enrichment without pre-heating)	128	47	11.4 (31)
a: 171 samples (63%) were positive by at least one method. b: TSC. c: cooked meat medium. d: After the heat pre-treatment, EDTA (20mM final concentration) was added and the sample incubated for 1 hour at 45°C before direct culture on TSC media.			

Figure 1. Distribution of the number of *C. perfringens* isolations from foal faeces by direct isolation methods (n = 271)

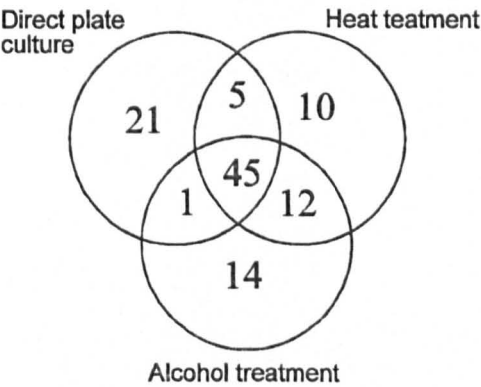


Figure 2. Distribution of the number of *C. perfringens* isolations from foal faecal samples by enrichment methods (n = 271)

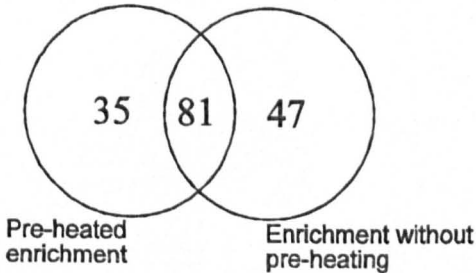
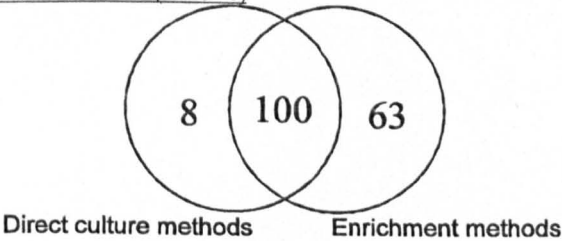


Figure 3. Comparison of distribution of the number of *C. perfringens* isolations from foal faecal samples by direct culture and enrichment methods (n = 271)



6.0 CHAPTER THREE

A MICROBIOLOGICAL SURVEY OF
FOAL DIARRHOEA

6.1 CONTENTS

6.1.1 List of Tables.....	57
6.2 Introduction.....	59
6.2.1 Microbiology of foal diarrhoea.....	59
6.3 Objectives.....	61
6.4 Methods and Materials.....	62
6.4.1 Collection of faecal samples from foals	62
6.4.2 Detection of Pathogens.....	62
6.4.3 Statistical Analyses	65
6.5 Results and Discussion.....	69

6.1.1 List of Tables

Table 6. Distribution of samples between Thoroughbred and other foals at stud or other premises.	67
Table 7. Distribution of samples between scouring and healthy foals of different ages.	67
Table 8. Distribution of the duration of scouring in foals before the faecal sample was collected	67
Table 9. Distribution of samples between scouring and healthy foals from different parts of the country.....	68
Table 10. Frequency of detection of <i>Strongyloides westeri</i> >2000 oocysts per gram of foal faeces	68
Table 11. Adjusted frequency of detection of <i>Strongyloides westeri</i> >2000 oocysts per gram of foal faeces.....	68
Table 12. Detection rates of pathogens in the faeces of scouring and healthy foals.	73
Table 13. Primary multivariate analysis of the association of pathogens with foals with diarrhoea compared with healthy foals not-in-contact with diarrhoea cases.	73
Table 14. Final multivariate analysis of the association of pathogens with foals	

with diarrhoea compared with healthy foals not-in-contact with diarrhoea cases.	74
Table 15. Multivariate analysis of the association of pathogens with foals with diarrhoea compared with all healthy foals.....	74
Table 16. The effect of different <i>C. perfringens</i> isolation methods on multivariate analysis of the association of pathogens with diarrhoea	74
Table 17. Univariate analysis of the numbers of <i>C. perfringens</i> cfu's per gram of faeces isolated from foals with diarrhoea and healthy foals not-in- contact with cases of diarrhoea	75
Table 18. Rate of detection (%) of pathogens in foals with a history of diarrhoea and which subsequently died (n=22).	76
Table 19. Age distribution of the isolation of <i>C. perfringens</i> from scouring foals which died.....	76
Table 20. Analysis of Rotavirus, <i>Clostridium perfringens</i> , and <i>Cryptosporidium</i> sp. in different parts of the country.	76
Table 21. Analysis of the interactions between <i>C. perfringens</i> and rotavirus	76

6.2 INTRODUCTION

6.2.1 Microbiology of foal diarrhoea

Many microbial causes of diarrhoea in foals have been proposed but their prevalence and significance is unclear or variably reported. Furthermore, there have been many case reports of suspected pathogens but there has not been a comprehensive survey of all potential pathogens other than of thoroughbreds in a stud environment (Dickie *et al.*, 1978; Tzipori, 1985b; Baker & Ames, 1987; Jones *et al.*, 1987; Myers *et al.*, 1987; Dart *et al.*, 1988; Dwyer *et al.*, 1990; Stubbings, 1990; Browning *et al.*, 1991; Holland *et al.*, 1991; Lyons *et al.*, 1991a; Lyons *et al.*, 1991b; Tschivdewahn *et al.*, 1991; Prescott & Hoffman, 1993). These studies calculated the percentage detection rates of the pathogens studied and in some a Chi squared analysis of the difference between healthy and scouring animals was used but they did not differentiate foals in-contact with cases of diarrhoea, from not-in-contact healthy foals. This means that isolates from foals with sub-clinical infections would have been counted as isolates from completely healthy animals, and thus assigned a greater non-pathogenic rôle as if they were not in-contact with cases and could not passively take up the pathogen and excrete it. This distinction is important when determining the significance of a microorganism, such as *Clostridium perfringens*, which is widespread and where a commensal rôle must be differentiated from a pathogenic rôle.

C. perfringens has previously been reported in association with a large number^{of} individual cases and outbreaks, in foals (see Chapter Two), but its prevalence and pathogenic rôle is unclear. *C. perfringens* enterotoxin, a virulence factor associated with several forms of *C. perfringens* diarrhoea in man and other animal species (see Chapter Two), may be a factor involved in the causation of foal diarrhoea. If the rôle of *C. perfringens* was investigated using only the traditional (human food poisoning) isolation methods (see Chapter Two), the investigation would be limited to one pathogenic mechanism. Therefore, it would be necessary to employ a range of methods to isolate *C. perfringens* in an epidemiological assessment

of the association of these bacteria with a complex disease such as foal diarrhoea. In this way, significant associations may be revealed without dependence on assumptions about the mechanisms by which *C. perfringens* might cause disease in relation to a sporulation event and the presence of large numbers of heat resistant endospores of a given maturity.

6.3 OBJECTIVES

The objectives were:

1. To obtain samples of faeces from thoroughbred and non-thoroughbred foals with and without diarrhoea throughout the United Kingdom.
2. To test them for the presence of *Salmonella* sp., rotavirus, 'thermophilic' *Campylobacter* sp., *Y. enterocolitica*, undifferentiated *E. coli*, *Cryptosporidium* sp., *S. westeri* and other helminths, by well recognised methods for each of the pathogens.
3. To detect the presence of *C. perfringens* by a variety of techniques, aimed at increasing the sensitivity of isolation of these bacteria which may be present as vegetative cells or endospores and which may be induced to germinate and grow by different treatments.
4. To estimate the prevalence of pathogens in foals with diarrhoea and to discover if there are statistically significant associations between any of these and diarrhoea by multivariate analysis.

6.4 METHODS AND MATERIALS

6.4.1 Collection of faecal samples from foals

During the latter part of 1991, and the whole of 1992 and 1993 samples of foal faecal samples (104, 267, and 272 respectively) were sent by veterinarians in response to a national call for material from cases of diarrhoea and healthy animals. Samples were requested from thoroughbred and non-thoroughbred foals, studs and other premises, foals of age 0 - 1 year old, foals with diarrhoea, healthy foals in-contact with cases of diarrhoea and healthy foals not-in-contact with cases of diarrhoea. The distribution of samples collected from different breeds on different types of premises is in Table 6; the age distribution of foals sampled is in Table 7; the distribution of duration of scouring at the time of sampling is in Table 8; and Table 9 details the distribution of samples by the area of the country from which the foals came.

Some samples, particularly those from East Anglia, were tested on the day they were taken. Most others, sent by post from further afield, were tested not more than two days after they were taken. Veterinarians and stud farm managers were instructed not to refrigerate samples because of its adverse effects on *C. perfringens* isolation.

6.4.2 Detection of Pathogens

Escherichia coli was isolated by culture on CLED medium (Oxoid, Unipath Ltd.) incubated aerobically at 37°C for 18 hours. Suspect colonies can be smooth, shiny, convex and yellow (lactose fermenting), or sometimes rough, lustreless and granular. Occasionally encapsulated variants produce mucoid colonies. *E. coli* was distinguished from other Gram-negative bacilli on the basis of the reactions in a combination of tests, primarily for motility, lactose utilisation, indole production in media containing tryptophan, citrate utilisation, decarboxylases, Voges-Proskauer reaction, fermentation of several carbohydrates, and urease production. Most of these tests are included in a commercially available kit (API20E; bioMérieux), in which the results are coded, and codes for the likely reactions of similar bacteria are compiled in

a data base, which was used to identify the organism. The kit was used according to the manufacturers instructions.

Thermophilic *Campylobacter sp.* were isolated on Campylobacter selective medium (Oxoid, Unipath Ltd.) incubated at 42°C in microaerobic chambers for 48 hours. *Campylobacter sp.* produces large, flat, glistening grey colonies on this medium, which were first Gram stained, and then tested for oxidase production. Oxidase-positive organisms, which showed typical microscopic morphology of curved or S-shaped Gram-negative bacilli were presumptively identified as *Campylobacter sp.*. Sensitivity to nalidixic acid distinguished between *C. lari* (resistant strains) and *C. jejuni / coli*.

Yersinia enterocolitica was isolated on Yersinia selective medium (Oxoid, Unipath Ltd.) incubated aerobically for up to 48 hours at 30°C. Bullseye colonies with a central magenta zone and translucent peripheral zone were screened for the presence of certain preformed enzymes using a commercially available kit (API Z; bioMérieux). If this test identified an organism as a presumptive *Y. enterocolitica*, it was confirmed by conventional biochemical tests using API20E kit (as above).

Salmonella sp. were isolated by enrichment in selenite broth with subculture on brilliant green agar and by direct inoculation of desoxycholate citrate agar (all three media; Oxoid, Unipath Ltd.); enrichment broths were incubated at 42°C for 24 hours before subculture onto solid media and culture on solid media was aerobic at 37°C for 48 hours with daily inspection for suspicious colonies. Most *Salmonella sp.* appear as red smooth convex colonies on brilliant green agar, and as colourless, translucent colonies on desoxycholate citrate agar but hydrogen sulphide producers may have black centred colonies. Colonies with these appearances were screened by the API Z (bioMérieux) kit for preformed enzymes, then confirmed by biochemical tests using the API20E kit (as above). Isolates confirmed as *Salmonella sp.* were sent to a reference laboratory to be serotyped.

Each faeces sample was cultured for *Clostridium perfringens* by three different direct and two separate enrichment methods to increase the chance of

isolating these bacteria in different physiological states (Chapter Two). The direct culture methods were:- (1) Direct culture - serial 10-fold dilutions of faeces in 2% w / v peptone water were spread plate inoculated onto tryptose sulphite cycloserine agar with added (0.1% w / v) lysozyme (TSC) and incubated in 10% CO₂ (v / v), 10% (v / v) hydrogen and 80% (v / v) nitrogen at 37°C for up to 4 days with daily inspection for suspicious colonies; dark colonies with lecithinase activity were enumerated to provide a presumptive count of the number of *C. perfringens* present. (2) Heat treatment - pre-treatment of faeces diluted to 10% (v / v) in sterile distilled water at 70°C for 20 minutes followed by the addition of sodium ethylene diamine tetracetic acid (EDTA) to 20 mM and further incubation at 45°C for 1 hour before culture on TSC plates as described above. (3) Alcohol shock treatment - pre-treatment of faeces diluted to 10% (v / v) in 10% (v / v) ethanol at 70°C for 10 minutes before culture on TSC as described above.

The enrichment methods were (1) Simple enrichment - suspension of faeces in Robertson's Cooked Media to 10% (v / v) followed by 18 hours anaerobic incubation at 37°C before subculture on TSC as described above. (2) Heat enrichment - as simple enrichment with the additional step of heating the faecal suspension at 70°C for 20 minutes before incubation.

C. perfringens was identified by its characteristic colony formation, its fermentation reactions towards lactose, sucrose, glucose, and salicin, and production of lecithinase and gelatinase (see Cowan, 1974 and Chapter Two).

Rotavirus was detected by Slidex Rota-Kit Monoclonal used according to the manufacturers instructions (bioMérieux). The kit is based on a latex agglutination test; a suspension of faeces (prepared according to the manufacturers instructions) was placed in two wells on a black card, then a latex suspension sensitised to the rotavirus group A antigen is added to one well, and a control latex suspension which is not sensitised to rotavirus, added to the other. The suspensions were mixed and rocked for two minutes, a positive result was characterised by agglutination occurring in the well with sensitised latex, but not in the other.

Cryptosporidium sp. were detected in auramine phenol stained smears by their characteristic green oocysts seen by fluorescent microscopy (Melvin & Healy, 1988; Austin *et al.*, 1990; Gnanasoorian, 1992), and, in equivocal cases, also by an immunofluorescence method utilising a labelled *Cryptosporidium sp.* antibody according to the manufacturers instructions (DetectIF *Cryptosporidium*; Northumbria Biologicals).

Helminths were detected by a modified McMaster method (M.A.F.F., 1985). A suspension of faeces was prepared in saturated sugar solution and placed in a McMaster slide. The slides were examined by light microscope and the number of oocysts per gram of faeces calculated.

6.4.3 Statistical Analyses

The data were analysed by standard means for case : control studies. They were recorded in Epi-info (Dean *et al.*, 1990) and transferred to EGRET (SERC 1989) for analysis. Initial univariate assessment of the association between any particular agent was followed by building logistic regression models, initially testing the inclusion of variables associated with disease at probability (p) = 0.2 or less, and then building the model by forward experimentation followed by backward stepwise model building with an acceptance level generally of $p < 0.05$.

The odds ratio is often used as an index of association in 2 x 2 contingency tables, and as the basis of log-linear models in larger and multidimensional tables. In this study it was used to estimate the association of a particular pathogen or factor with diarrhoea in foals.

When calculating odds ratio estimators, if under condition 1, the probabilities of failures and successes are respectively P_1 and $(1 - P_1)$, and if these probabilities are P_2 and $(1 - P_2)$ under condition 2, then, the odds ratio, R , between the two conditions is defined as $R = [P_1 / (1 - P_1)] / [P_2 / (1 - P_2)]$. In a 2 x 2 table, if the observed frequencies corresponding to these probabilities are a , b , c , and d , then the traditional estimate of R is $R = [a / b] / [c / d] = ad / bc$. Since this estimator is undefined if either b or c is zero, a number of authors have proposed adding small constants to the

cell frequencies (Woolf, 1955; Haldene, 1956; Jewell, 1986). Jewell proposed the estimator $R = ad / [(b+1)(c+1)]$ which is much less biased than the traditional estimator. A recent study comparing several such estimators concluded that Jewell's modification was generally satisfactory (Walter & Cook, 1991).

In the present study, the computing software yielded satisfactory results in the logistic regression model, except for the effects of *Strongyloides westeri* in more than 2000 oocysts per gram of faeces, for which the algorithm failed to converge. The observed data for *S. westeri* are summarised in Table 10. Since all 24 foals in whose faeces *S. westeri* was detected had diarrhoea, there is a clear *a priori* association between *S. westeri* and diarrhoea. The failure of the algorithm to converge when *S. westeri* was included in the model was clearly due to the zero frequency in the cell containing healthy foals with *S. westeri*, (i.e. the lack of healthy foals with *S. westeri*).

To overcome this computational problem, the basic raw data were adjusted so as to modify the frequencies of foals with and without *S. westeri* to that shown in Table 11. The effect of this adjustment on the estimate of the odds ratio, R , will be the same as using $R = ad / [(b+1)(c+1)]$, as recommended by Jewell (1986). With the large frequencies elsewhere in the data, estimates of all other parameters in the model should be changed only negligibly by the above minor adjustment. In particular, the effect of the adjustment on the estimate of the odds ratio for *S. westeri* is to make it conservative, i.e. an underestimate. The same is also true for the significance test for this parameter. The adjusted data were then fitted in the multivariate model.

Since the fit of the multivariate model was not significantly affected for rotavirus, *C. perfringens* and *Cryptosporidium* sp. by the inclusion of *S. westeri*, analysis of the method of isolation of *C. perfringens* and the rôle of pathogens in different parts of the country utilised a model without *S. westeri* to conserve computer processing power.

Table 6 Distribution of samples between Thoroughbred and other foals at stud or other premises.					
Premises	Breed	Diarrhoea	Number of foal faecal samples	Number of sampling groups	Range of sampling group size (mean)
Studs	Thoroughbred	+	209	73	1-17 (1.9)
		-	155	14	1-11 (5.9)
	Other breeds	+	41	12	1-16 (2.6)
		-	60	8	1-21 (4.7)
Other Premises	Thoroughbred	+	43	42	1-3 (1.2)
		-	2	2	1-2 (1.5)
	Other breeds	+	128	104	1-4 (1.1)
		-	5	3	1-2 (1.3)
Sampling group: samples taken from foals at a particular location separated by an interval of 3 weeks from any other sample taken from that location.					

Table 7 Distribution of samples between scouring and healthy foals of different ages.		
Age of foals	Samples from healthy foals	Samples from scouring foals
≤ 1 week of age	10	61
>1 to 4 weeks of age	30	173
> 4 weeks of age	182	187

Table 8 Distribution of the duration of scouring in foals before the faecal sample was collected.	
Length of scouring (weeks)	Samples from scouring foals
<1	375
1	21
2	6
3	2
4	4
5	1
6	1
7	1
≥8	12

Table 9 Distribution of samples between scouring and healthy foals from different parts of the country.		
Area of country	Samples from healthy foals	Samples from scouring foals
East Anglia	163	147
South of England	29	132
North of England and Scotland	0	37
Midlands and Wales	30	105

Table 10 Frequency of detection of <i>Strongyloides westeri</i> > 2000 oocysts per gram of foal faeces.			
Number of foals	Healthy not-in-contact foals	Scouring foals	Row Total
<i>S. westeri</i> >2000 oocysts / gram of faeces not present	109	341	450
<i>S. westeri</i> >2000 oocysts / gram of faeces present	0	24	24
Column Total	109	365	474

Table 11 Adjusted frequency of detection of <i>Strongyloides westeri</i> > 2000 oocysts per gram of foal faeces.			
Number of foals	Healthy not-in-contact foals	Scouring foals	Row Total
<i>S. westeri</i> >2000 oocysts / gram of faeces not present	109	342	451
<i>S. westeri</i> >2000 oocysts / gram faeces present	1	24	25
Column Total	110	366	476

6.5 RESULTS AND DISCUSSION

Rates of detection of pathogens from the faeces of healthy and scouring foals are detailed in Table 12. Very few cases of diarrhoea yielded *Salmonella* sp. and these few were largely isolated from one distinct outbreak in non-thoroughbred horses at stud. The serotype isolated was *S. typhimurium*. The percentage isolation rate for thermophilic *Campylobacter* sp. (7.5% *C. coli* / *jejuni*; 0.5% *C. lari*), *Yersinia enterocolitica* and *Escherichia coli* did not seem markedly different between scouring and healthy foals. The healthy foals were split into two groups; those which were in-contact with cases of diarrhoea, and those which were not-in-contact. This enabled an estimation of the subclinical infection rate and normal healthy carriage rate, respectively. It was expected that comparison between scouring foals and foals not-in-contact with diarrhoea would give a more accurate estimation of association of each pathogen with diarrhoea; in-contact healthy animals may shed the pathogen more frequently than not-in-contact animals because of the potential for ingestion and passive carriage of the pathogen. Univariate analysis indicated a statistically significant association with diarrhoea for rotavirus, *Cryptosporidium* sp., *S. westeri*, and *C. perfringens*. These organisms were included in the logistic regression model and the other organisms were excluded by the multivariate analysis.

Multivariate analysis of scouring and not-in-contact healthy foals showed that rotavirus, *Cryptosporidium* sp. and *C. perfringens* were significantly associated with diarrhoea (Table 13). The association of *C. perfringens* was particularly strong in foals of less than one week of age (Table 14); a similar variation in association with age was not detected for rotavirus, *Cryptosporidium* sp., or *S. westeri*. At least one of the significantly associated pathogens were isolated from 75% of foals with diarrhoea (Table 12).

The detection rates between healthy in-contact and not-in-contact foals were compared (Table 12) to assess if contact with diseased animals affected the carriage rate. The detection rate was greater in healthy in-contact foals than in not-in-contact foals for *Cryptosporidium* sp., rotavirus and *C. perfringens*. In spite of this, a

multivariate analysis of association between pathogens and diarrhoea using comparison with the healthy in-contact foals alone showed that rotavirus and *C. perfringens* were significantly associated with disease (odds ratio and p value respectively of 2.9, 0.005 for rotavirus; 3.0, <0.001, for *C. perfringens*).

The association of *C. perfringens* with scouring foals is greater in animals of less than one week of age (odds ratio = 30.77; $p < 0.001$; multivariate analysis with not-in-contact controls), and slightly less in foals aged 2 to 4 weeks (odds ratio = 10.77), but not significantly different from younger foals. However, although *C. perfringens* is still associated with diarrhoea in foals greater than 4 weeks of age (odds ratio = 4.00), the odds ratio is significantly different from the association with diarrhoea in foals less than 4 weeks of age (Table 14). A similar analysis, with all healthy foals as controls, still resulted in the same organisms being significantly associated with diarrhoea, but with the odds ratio values reduced (Table 15). This demonstrated how in-contact carriers of pathogens can appear to reduce the significance of pathogenic bacteria.

Different methods of isolation of *C. perfringens* were used because each would favour recovery of these bacteria in different states ranging from vegetative cell to endospore. Combination of these methods would be likely to give an overall increased sensitivity of isolation. This proved to be so because there was at least one sample positive for each method when the other methods were negative; 20% of the *C. perfringens* isolations were made by one of the five methods alone. Furthermore, the association with diarrhoea was greater with heat enrichment than any other method (Table 16) indicating the possibility of an association between diarrhoea and heat resistant endospore formation. However, isolation of *C. perfringens* by alcohol treatment was negatively associated with diarrhoea.

Analysis of isolation methods where viable counts were made did not reveal a particular association between diarrhoea and large numbers of *C. perfringens*. All numbers (apart from 10^5 - 10^6 per gram) of *C. perfringens* isolated by direct culture were associated with diarrhoea but there was no association between diarrhoea and

recovery of large numbers of these bacteria by heat or alcohol pre-treatment methods (Table 17).

Multivariate analysis revealed that only *S. typhimurium* and *C. perfringens* isolation were significantly associated with diarrhoea with a fatal outcome for samples taken at post mortem examination or antemortem. The percentage of positive foals from samples taken antemortem was greater for these pathogens, which suggested that the association was not falsely created by post mortem proliferation by bacteria which had not acted as pathogens (Table 18). A large proportion of foals that died were less than 1 week old, of which 75% were positive for *C. perfringens*. Similarly, a large proportion of *C. perfringens* isolations from foals which died were less than 1 week of age (Table 19). These results suggest that fatal *C. perfringens* diarrhoea tends to occur in very young foals.

Frank blood in the faeces sample was noted only four times in scouring foals on each occasion. Two of these were post mortem samples and *C. perfringens* was isolated from both.

Analysis of the association between diarrhoea in relation to breed of foal and type of premises, for rotavirus, *C. perfringens*, and *Cryptosporidium* sp., revealed **no change** in association with diarrhoea. Too few samples from healthy foals not-at-stud were provided to separately estimate if there was any association between pathogens and diarrhoea outside of studs.

Most of the samples submitted were from foals in the first 4 months of life; the peak foal age was one month. Consequently, there were insufficient samples from the older age groups to detect statistically an age related trend in association between specific pathogens and diarrhoea.

The relatively small group sizes (Table 6) particularly from non-thoroughbred foals and the sporadic nature of sample submission may have contributed to a failure to detect any pathogen specific outbreaks. Just over 25% of foals with diarrhoea were being treated with antibiotics at the time the sample was taken and antibiotic treatment was associated with diarrhoea ($p < 0.001$). However, taking this association

into account, there was no relationship (inverse or direct) between *C. perfringens* isolation and antibiotic treatment.

Other than East Anglia, there were too few samples to assess if there was an association between any of the pathogens and diarrhoea occurring in each area. If samples from all of the areas outside of East Anglia were amalgamated and treated as one area then rotavirus and *C. perfringens* were associated with diarrhoea in East Anglia and, independently, in the rest of the country as well (Table 20) when comparing scouring foals with healthy not-in-contact foals. *Cryptosporidium sp.* was associated with diarrhoea only in East Anglia. Failure to detect an association between scouring foals and *Cryptosporidium sp.* was probably a result of sampling structure, and hence computational difficulty, such as the complete absence of this agent from healthy animals.

The duration of diarrhoea at the time of sampling for the vast majority of samples was less than one week (Table 8). There was no greater association between the isolation of *C. perfringens* from samples taken at longer than one weeks duration of diarrhoea compared with less than one week duration.

There was no evidence of an interaction between rotavirus and *C. perfringens* (Table 21) in association with diarrhoea. This was borne out by a lack of improvement of the fit of the final multivariate model when an interaction term between *C. perfringens* and rotavirus was added. Consequently, these results provided good evidence that *C. perfringens* is independently associated with diarrhoea but if these bacteria are a cause of scouring the mechanism of pathogenicity was unclear.

Table 12 Detection rates of pathogens in the faeces of scouring and healthy foals. (Number tested)

Organism	Number tested	Percentage positive faeces overall	Percentage positive faeces from foals with diarrhoea	Percentage positive faeces from healthy foals in-contact with diarrhoea cases	Percentage positive faeces from healthy foals not-in-contact with diarrhoea cases
<i>S. typhimurium</i>	643	2	1 (421)	9 (102)	0 (120)
<i>Campylobacter sp.</i>	643	8	8 (421)	5 (102)	8 (120)
<i>Yersinia enterocolitica</i>	643	<1	1 (421)	0 (102)	0 (120)
<i>Escherichia coli</i>	643	94	95 (421)	92 (102)	91 (120)
Rotavirus	637	16	22 (417)	11 (102)	1 (118)
<i>Cryptosporidium sp.</i>	637	17	19 (417)	18 (102)	7 (118)
<i>Strongyloides westeri</i> overall	574	6	6 (366)	11 (99)	2 (109)
<i>Strongyloides westeri</i> >2000 oocysts/g faeces	576	3	5 (367)	2 (99)	1 (110)
Other parasites	574	5	4 (366)	16 (99)	1 (109)
<i>Clostridium perfringens</i> overall	643	46	57 (421)	33 (102)	19 (120)
<i>Clostridium perfringens</i> in foals ≤ 1week of age	71	76	80 (61)	57 (7)	33 (3)
<i>Clostridium perfringens</i> in foals 2-4weeks of age	203	57	59 (173)	56 (16)	36 (14)
<i>Clostridium perfringens</i> in foals >4 weeks of age	369	34	47 (187)	27 (79)	17 (103)

Table 13 Primary multivariate analysis of infection rates in foals with diarrhoea compared with healthy foals not-in-contact with diarrhoea cases.*

Organism	Odds Ratio	p value	95% Confidence limits
Intercept (α term)	1.1	0.332	0.87 - 1.52
<i>Clostridium perfringens</i>	7.4	<0.001	3.73 - 10.56
Rotavirus	40.5	<0.001	5.38 - 289.70
<i>Cryptosporidium sp.</i>	3.2	<0.001	1.76 - 6.17

* Primary analysis which did not include *Strongyloides westeri* as a variable or the stratification of *C. perfringens* by age.

Table 14 Final multivariate analysis of the association of pathogens with foals with diarrhoea compared with healthy foals not-in-contact with diarrhoea cases.

Organism	Odds Ratio	p value	95% Confidence limits
Intercept (α term)	1.00	0.853	0.72 - 1.31
<i>Clostridium perfringens</i> in foals \leq 1 week of age	30.77	<0.001	4.11 - 230.40
<i>Clostridium perfringens</i> in foals >1 to 4 weeks of age	10.77*	-	-
<i>Clostridium perfringens</i> in foals > 4 weeks of age	4.00*	-	-
Rotavirus	42.28	<0.001	5.73 - 311.80
<i>Cryptosporidium</i> sp.	3.30	<0.001	1.72 - 6.33
<i>Strongyloides westeri</i> >2000 oocysts / g of faeces	7.79	0.052	1.00 - 61.68

* Odds Ratio derived from multiplication of age.*C. perfringens* term and *C. perfringens* term.

Table 15 Multivariate analysis of the association of pathogens with foals with diarrhoea compared with all healthy foals.

Organism	Odds Ratio	p value	95% Confidence limits
Intercept (α term)	0.65	0.001	0.50 - 0.84
<i>Clostridium perfringens</i> in foals \leq 1 week of age	12.39	<0.001	4.27 - 35.93
<i>Clostridium perfringens</i> in foals >1 to 4 weeks of age	7.01*	-	-
<i>Clostridium perfringens</i> in foals > 4 weeks of age	2.49*	-	-
Rotavirus	6.23	<0.001	3.15 - 12.34
<i>Cryptosporidium</i> sp.	2.06	<0.001	1.38 - 3.10
<i>Strongyloides westeri</i> >2000 oocysts / g of faeces	6.20	0.018	1.36 - 28.19

* Odds Ratio derived from multiplication of age.*C. perfringens* term and *C. perfringens* term.

Table 16 The effect of different *Clostridium perfringens* isolation methods on multivariate analysis of the association of pathogens with diarrhoea.

<i>Clostridium perfringens</i> isolation method	Odds Ratio	p-value	95% Confidence limits
Direct Plate	2.0	<0.001	1.38 - 2.93
Alcohol Treatment	0.8	<0.001	0.05 - 2.35
Heat Treatment	2.9	<0.001	1.66 - 5.13
Pre-heated Enrichment	4.7	<0.001	2.29 - 9.80
Enrichment without pre-heating treatment	3.0	<0.001	2.01 - 4.61

Table 17 Univariate analysis of the numbers of *C. perfringens* cfu's per gram of faeces isolated from foals with diarrhoea and healthy foals not-in-contact with cases of diarrhoea.

Isolation Method / Number of colony forming units per gram of faeces*	Number of positive faecal samples	Number of positive faecal samples in faeces from not-in-contact foals	Odds Ratio	p - value	95% Confidence limits
Direct plate culture 10 ³	56	7	2.49	0.025	1.05 - 6.22
Direct plate culture 10 ⁴	22	0	infinity	0.006	~
Direct plate culture 10 ⁵	18	1	6.05	0.048	0.84 - 123.49
Direct plate culture 10 ⁶	12	2	1.78	0.456	0.36 - 11.96
Direct plate culture >10 ⁶	14	0	infinity	0.027	~
Heat treatment 10 ³	46	3	4.82	0.005	1.40 - 19.87
Heat treatment 10 ⁴	17	1	5.38	0.070	0.74 - 110.06
Heat treatment 10 ⁵	9	2	1.18	0.840	0.22 - 8.32
Heat treatment 10 ⁶	6	0	infinity	0.157	~
Heat treatment >10 ⁶	8	1	2.35	0.412	0.05 - 18.92
Alcohol treatment 10 ³	38	1	3.62	0.195	0.47 - 76.46
Alcohol treatment 10 ⁴	14	1	1.27	0.824	0.15 - 28.03
Alcohol treatment 10 ⁵	7	0	infinity	0.411	~
Alcohol treatment 10 ⁶	4	1	0.29	0.277	0.02 - 7.87
Alcohol treatment >10 ⁶	3	0	infinity	0.590	~

* : The number of colony forming units were rounded upto the nearest power of ten.

~ : 95% confidence limits could not be calculated.

Table 18 Rate of detection (%) of pathogens in foals with a history of diarrhoea and which subsequently died (n = 22).

Organism	All cases (Odds Ratio; p-value; CL)	Antemortem samples (n = 6) positive (%)
<i>Salmonella typhimurium</i>	9 (13.4; 0.005; 1.69 - 62.49)	17
Thermophilic <i>Campylobacter</i> sp.	14 (NS)	33
<i>Escherichia coli</i>	100 (NS)	100
Rotavirus	10 (NS)	17
<i>Cryptosporidium</i> sp.	14 (NS)	33
<i>S. westeri</i>	0 (NS)	0
<i>Clostridium perfringens</i>	64 (2.7; 0.047; 1.20 - 7.28)	83

NS: not significant. CL: 95% Confidence Limits.

Table 19 Age distribution of the isolation of *C. perfringens* from scouring foals which died.

Age of foals	Number of foals tested	Number of foals positive for <i>C. perfringens</i>	Number of foals negative for <i>C. perfringens</i>
≤ 1 week	8	6	2
> 1 week	14	8	6

Table 20 Analysis of Rotavirus, *Clostridium perfringens*, and *Cryptosporidium* sp. in different parts of the country.

Organism	East Anglia (scouring vs. not-in-contact healthy foals) Odds Ratio (p-value; confidence interval)	East Anglia (scouring vs. all healthy foals) Odds Ratio (p-value; confidence interval)	Rest of the country (scouring vs. all healthy foals) Odds Ratio (p-value; confidence interval)
Rotavirus	32.1 (0.001; 3.94 - 242.60)	4.5 (<0.001; 1.85-11.21)	4.1 (0.003; 1.44 - 12.06)
<i>Clostridium perfringens</i>	10.5 (<0.001; 4.83 - 17.44)	7.0 (<0.001; 4.07-11.97)	1.7 (0.033; 1.05 - 3.05)
<i>Cryptosporidium</i> sp.	3.6 (<0.001; 1.74 - 6.95)	2.6 (0.002; 1.51 - 4.62)	1.1 (0.825; 0.69 - 1.87)

Insufficient samples were received to make the analysis of scouring vs. not-in-contact foals from areas outside of East Anglia.

Table 21 Analysis of the interactions between *C. perfringens* and rotavirus.

<i>C. perfringens</i>	Rotavirus	Number of cases (scouring foals)	Number of controls (all healthy foals)	Odds Ratio	p-value	Confidence limits
-	-	141	157	1.0	-	-
-	+	39	7	6.2	<0.001	2.56 - 15.73
+	-	185	51	4.0	<0.001	2.70 - 6.05
+	+	52	5	11.6	<0.001	4.28 - 33.94

7.0 CHAPTER FOUR

DETECTION OF THE *CLOSTRIDIUM PERFRINGENS*
ENTEROTOXIN IN FAECES AND ITS
GENETIC CLONING AND CHARACTERISATION FROM AN
EQUINE ISOLATE

7.1 CONTENTS

7.1.1 List of Tables.....	79
7.1.2 List of Figures	79
7.2 Introduction	80
7.2.1 Virulence factors of <i>Clostridium perfringens</i>	80
7.2.2 The <i>Clostridium perfringens</i> enterotoxin	80
7.3 Objectives	82
7.4 Methods and Materials	83
7.4.1 Enterotoxin detection	83
7.4.2 Bacteria.....	84
7.4.3 Media	84
7.4.4 Purification of <i>C. perfringens</i> DNA	85
7.4.5 General molecular methods.....	85
7.4.6 Agarose gel electrophoresis	85
7.4.7 SDS-PAGE	86
7.4.8 PCR amplification of a putative enterotoxin gene fragment from an equine strain of <i>C. perfringens</i>	87
7.4.9 Cloning of the putative enterotoxin gene into plasmid pTrc 99A.....	88
7.4.10 Detection of recombinant pTrc 99A.....	89
7.4.11 Induction of enterotoxin gene expression	90
7.4.12 Single stranded DNA sequencing	90
7.4.13 Statistical Analyses	95
7.5 Results and Discussion	96

7.1.1 List of Tables

Table 22. Detection of enterotoxin by RPLA directly in faeces and in
sporulated culture supernatant fractions of isolates of
C. perfringens 101

7.1.2 List of Figures

FIGURE 4. Photograph of an agarose gel with PCR products of DNA
extracts of *Clostridium perfringens* B3550.5, NCTC8239, and
E. coli JM105 recombinant containing the enterotoxin gene..... 98

FIGURE 5. Photograph of an SDS-PAGE gel of the *E. coli* JM105
p*Trc* 99A enterotoxin gene recombinant and non-recombinant
culture supernatant fractions..... 99

FIGURE 6. Sequence of *Clostridium perfringens* B3550.5 enterotoxin
gene 100

7.2 INTRODUCTION

7.2.1 Virulence factors of *Clostridium perfringens*

Different strains of *C. perfringens* utilising different pathogenic mechanisms are often responsible for distinct syndromes in several mammalian species (Hobbs *et al.*, 1953; Shann *et al.*, 1979; Jewkes *et al.*, 1981; Stringer, 1985; Tzipori, 1985b; Van Kessel *et al.*, 1985; Walker, 1985; Niilo, 1987; Van Baelan & Devriese, 1987; Borriello, 1988; Traub-Dargatz *et al.*, 1988; Collins *et al.*, 1989; Johnson *et al.*, 1992). Many of these syndromes result from the elaboration of a variety of toxins in the gastrointestinal tract which have an aggressive action causing severe and often fatal tissue damage (Shann *et al.*, 1979; Niilo, 1980; Borriello & Carman, 1985; Tzipori, 1985b; Walker, 1985). Strains which produce an enterotoxin which stimulates hypersecretion of fluid are a cause of food poisoning (Stringer, 1985) and infectious diarrhoea in institutionalised populations (Borriello *et al.*, 1985) which is milder than the necrotic enteric disease associated with the more aggressive toxins.

The use of a variety of isolation techniques to increase the chances of culturing *C. perfringens* existing as vegetative cells or endospores with different germination properties (see Chapter 2), has identified *C. perfringens* as an important potential cause of diarrhoea in foals (see Chapter 3). *C. perfringens* was also the pathogen associated with the largest proportion of diarrhoea with a fatal outcome from an identifiable infectious disease. The mechanism(s) of pathogenesis is / are unknown, however, although it was clear that the majority of disease associated with *C. perfringens* was not of the life threatening kind. The virulence factor, *C. perfringens* enterotoxin, is often associated with the milder forms of diarrhoea (Borriello *et al.*, 1985; Stringer, 1985) and could be associated with diarrhoea in foals.

7.2.2 The *Clostridium perfringens* enterotoxin

Peak enterotoxin production coincides with sporulation and large numbers of endospores are found in the faeces of food-borne cases of disease. Enterotoxin has been considered a component of the endospore coat (Friebe & Duncan, 1973), but

since strains which do not produce enterotoxin can sporulate it may not be an essential endospore component. Also it has been shown that the enterotoxin gene may be expressed constitutively from a weak promoter during the exponential phase of vegetative growth (Granum *et al.*, 1984; Goldner *et al.*, 1986). It is now thought that its gene is transcribed from a developmentally regulated promoter which requires the product of an unknown (stage I or stage II) sporulation gene for activation. Therefore, the enterotoxin gene may not be involved in the process of sporulation as such, but may be under co-ordinate control by transcriptional factors in response to an environmental stimulus. On the other hand, it may be that an enterotoxin equivalent, equally essential for sporulation, is present in 'non-enterotoxigenic' *C. perfringens*. Large amounts of enterotoxin are produced in the cytoplasm and its accumulation can lead to aggregation, inclusion body formation and trapping of small amounts of enterotoxin during endospore biogenesis (Rood & Cole, 1991). Establishment of the dependence of sporulation on enterotoxin awaits toxin mutagenesis studies.

Partial DNA sequences of this toxin^{gene} were initially obtained because of difficulties in cloning the whole gene (Hanna *et al.*, 1989; Iwanejko *et al.*, 1989; Van Damme-Jongsten *et al.*, 1989). Recently, however, the whole gene was cloned and sequenced but some regions of the complete sequence were not homologous with the previously published sequences for gene fragments (Czeczulin *et al.*, 1993).

The native enterotoxin contains all 20 amino acids and comprises 320 residues to give a molecular weight of 35,391 (Czeczulin *et al.*, 1993). The publication of the complete sequence has provided the opportunity to look for and characterise the enterotoxin gene in equine isolates of *C. perfringens*.

7.3 OBJECTIVES

The objectives were:

1. To assess the association between foal diarrhoea and (a) enterotoxin in faeces detected antigenically by reverse passive latex agglutination (RPLA) and (b) enterotoxin production by *C. perfringens* isolates detected by RPLA.
2. To verify enterotoxin production by a selected equine isolate by neutralisation of its activity for Vero cells by specific antiserum.
3. To attempt to clone and sequence the gene for enterotoxin production from an equine isolate and assess its relatedness to the gene sequenced from the human isolate.
4. To express the equine enterotoxin gene in a recombinant *Escherichia coli*, detect its presence by RPLA and specifically neutralisable verotoxicity, and compare recombinant expression with expression from the parent *C. perfringens*.

7.4 METHODS AND MATERIALS

7.4.1 Enterotoxin detection

Isolates of *C. perfringens* cultured in cooked meat media (Oxoid, Unipath Limited) were encouraged to sporulate by sub-inoculation into Duncan and Strong medium (Duncan and Strong, 1968) and incubation in 10% CO₂ (v / v), 10% (v / v) hydrogen and 80% (v / v) nitrogen for 18 hours at 37°C; the culture supernate was recovered and the enterotoxin was detected by reverse passive latex agglutination (RPLA; Oxoid, Unipath Limited). The supernatant fraction was titrated by doubling dilutions, in duplicate, in a V-well microtitre tray. Latex particles sensitised with purified immunoglobulin taken from rabbits immunised with *C. perfringens* enterotoxin were added to each well and these agglutinated if the *C. perfringens* enterotoxin was present. A control reagent was used in parallel, which consisted of latex particles sensitised with non-immune rabbit globulins. The faecal samples were also examined directly by the same method, following an initial dilution with sample buffer and then centrifugation (relative centrifugal force of 8000 g, for 20 minutes) to remove large particles. All methods for sample preparation, culture preparation and enterotoxin detection followed the manufacturers recommendations, with the exception of the titration of the test samples. Samples were first screened using one test well and one control well. Any positive samples were then fully titrated in duplicate. A final positive result comprised the presence of agglutination of the latex particles in a sample dilution of 1 / 8 (includes addition of an equal volume of latex).

Cytotoxicity of the culture supernatants was determined by a Vero cell cytotoxicity assay (Borriello *et al.*, 1984), employing a polyclonal *C. perfringens* enterotoxin antiserum produced by Biogenesis Limited. Briefly, mono-layers of Vero cells were cultured in flat well microtitre trays and serial doubling dilutions of faecal suspensions (starting dilution of 1 / 100 to reduce the intrinsic cytotoxic nature of equine faeces), were added to two parallel sets of wells with polyclonal *C. perfringens* enterotoxin antiserum added to one set of wells. The degree of cytotoxicity, rounding and destruction of cells, was compared in the two sets of wells

and the extent of neutralisation determined. A positive result, was characterised by the presence of cytotoxicity which could be neutralised by at least four-fold.

7.4.2 Bacteria

Clostridium perfringens isolates (549) from 240 of 421 foals with diarrhoea and 98 isolates from 53 of 222 healthy foals were tested for enterotoxin production (one representative isolate per positive isolation method per sample - see Chapter Three). *C. perfringens* NCTC8239, a human isolate from which the complete enterotoxin gene was originally cloned and sequenced (Czeczulin *et al.*, 1993), served as a control for the polymerase chain reaction for the enterotoxin gene. *Escherichia coli* JM105 was used for the plasmid cloning and *E. coli* TG1 for M13 subcloning of the enterotoxin gene.

7.4.3 Media

The media required to culture the strains of bacteria used in this study (see section 7.4.2) include Luria Bertani (LB) broth and agar. LB broth was made from 10 g bactotryptone (Oxoid; Unipath Ltd.), 5 g yeast extract (Oxoid; Unipath Ltd.) and 5 g sodium chloride (Sigma) with distilled water added to 1 litre. LB agar plates require an additionally included 12 g technical agar (Oxoid; Unipath Ltd.). Both were sterilised at 121°C and 115 lbs for 15 minutes.

Minimal media was made from 6.01 g of technical agar (Oxoid; Unipath Ltd.) dissolved in 360 ml by autoclaving at 121°C and 115 lbs for 15 minutes followed after cooling by 80 ml of sterile 5 x M9 salts (Sigma), 400 µl of sterile 20% (w / v) glucose, 40 µl of sterile 1% (w / v) thiamine and 67 µl of sterile 1 M magnesium sulphate before the plates were poured.

SOB medium was prepared with 20 g bacto-tryptone (Oxoid; Unipath Ltd.), 5 g bacto-yeast extract (Oxoid; Unipath Ltd.) and 0.5 g sodium chloride in 950 ml of distilled water, which was autoclaved at 121°C and 115 lbs for 15 minutes. Just before use, 5 ml of sterile 2 M magnesium chloride was added. SOB agar was made in a similar manner with the addition of 12 g of technical agar (Oxoid; Unipath Ltd.).

7.4.4 Purification of *C. perfringens* DNA

Overnight cultures of *C. perfringens* in Fastidious Anaerobe Broth (20 ml volumes; Oxoid) at 37°C in an atmosphere of 10% (v / v) CO₂, 10% (v / v) hydrogen, and 80% (v / v) nitrogen, were centrifuged at 10,000 g for 7 minutes at 4°C. The cells were resuspended in 1 ml of 8 M guanidine thiocyanate, boiled for 15 minutes, allowed to cool and then centrifuged at 10,000 g for 7 minutes. DNA was recovered from the supernatant fraction using the Magic DNA Clean-up System (Promega). This is based on the removal of salts, enzymes and small (≤ 175 bp) DNA fragments by passing the sample through a resin column with differential resin binding and elution; it was primarily used according to the manufacturers instructions but purified DNA was eluted at 90°C rather than 65-70°C.

7.4.5 General molecular methods

Details of general methods for and underlying principles of plasmid propagation and purification, PCR, cloning, subcloning, restriction enzyme digestion, agarose gel electrophoresis, ligation, transformation, selection of recombinants, inducement of recombinants to produce enterotoxin, polyacrylamide gel electrophoresis, M13mp19 phage propagation, and phage DNA purification were as described by Sambrook *et al.*, 1989.

7.4.6 Agarose gel electrophoresis

Agarose gel electrophoresis was used to estimate the size and concentration of PCR products, plasmids and phage DNA by comparison with the migration and concentration of Lambda phage DNA digested with Hind III (Sigma). Samples were mixed with gel loading solution (Sigma), then loaded into wells of a 0.7% (w / v) agarose gel, which were run in an LKB GNA100 apparatus (Pharmacia) with 0.4 M Tris acetate 0.01 M EDTA buffer pH 8.3 (Sigma) using a power supply of 65 Volts for 80 minutes. Ethidium bromide was added to the gels at 4.3×10^{-6} % (w / v) so that DNA could be seen by the fluorescence given off when the gel was placed on an ultra-violet transilluminator. A photographic record of the gel was made using a video copy processor (Mitsubishi Electrical (UK) Limited).

The resolving power of agarose gels is a function of the concentration of dissolved agarose. The migration rate of nucleic acids through agarose gels is additionally dependent upon the molecular size (for linear fragments), conformation, and voltage gradient.

7.4.7 SDS-PAGE

Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and silver staining were used to determine whether the recombinant *E. coli* produced a protein not seen with the non-recombinant *E. coli* which was of the size expected of enterotoxin. The discontinuous buffer system of Laemmli (1970) was used. The percentage of acrylamide in the separating gel was 12% (w / v) and contained 1.5 M Tris-HCl, pH 8.8 and 0.1 % (w / v) SDS. The stacking gel was 4% (w / v) and contained 0.5 M Tris-HCl, pH 6.8, and 0.1 % (w / v) SDS. The acrylamide monomers were polymerised by a free radical chain reaction, initiated by 0.05% (w / v) ammonium persulphate and activated by 0.1% (v / v) *N,N,N,N*-tetramethylethylenediamine (TEMED), into linear chains and the linking of these chains with *N,N*-methylenebisacrylamide (bis). The gel was poured in a Protean II apparatus according to the manufacturers instructions (BioRad), and 20 µl of each sample was premixed with an equal volume of sample buffer [0.5 M Tris-HCl pH 6.8, 2% (w / v) SDS, 5% (w / v) 2-mercaptoethanol], boiled for 2 minutes and then loaded in the wells. Standard mixtures of proteins of known molecular weight (M5630 and M5505; Sigma) were loaded in separate wells. The reservoirs were filled with a buffer of 0.025 M Tris-glycine, pH 8.3 and 0.1% (w / v) SDS and the gel was run with a power supply of at 25 mA for 3 hours.

The concentration of acrylamide and the ratio of acrylamide to bis determine the pore size of the resultant three-dimensional network and hence its sieving effect on proteins of different sizes. The direction and speed of migration of proteins undergoing electrophoresis depends on the charge of the protein, and the strength of the electric field which directly affects mobility and also influences frictional forces which oppose migration. The SDS (sodium dodecyl sulphate) anionic detergent,

complexes with protein to form a rod-like structure of uniform size to negative charge ratio; some regions of polypeptide bind more detergent than others, however. With the exception of some structural proteins, protein-SDS complexes are soluble and will migrate through a polyacrylamide gel towards the anode at a rate inversely proportional to the logarithm of their molecular weight. This relationship may be used to determine the molecular weight of an unknown protein by comparison with the migration of proteins of known molecular weight. A kit performed according to the manufacturers instructions (Sigma) was used to stain the protein bands with silver.

7.4.8 PCR amplification of a putative enterotoxin gene fragment from an equine strain of *C. perfringens*

The primers were derived from the sequence published for a human isolate (Czeczulin *et al.*, 1993) with the intention of cloning the PCR product into the expression vector pTrc 99A (see section 7.4.9).

The N terminus primer had the sequence 5' TAT ACC ATG GTT AGT AAC AAC AAT TTA AAT CC substituting a G for a C at the first position of the second codon; this would have the effect of substituting a valine for a leucine residue in any product but was used to create the 'in frame' *Nco* I site compatible with the multiple cloning site of pTrc 99A. The C terminus primer on the complementary strand had the sequence 5' AT GGA TCC ATA TTA AAA TTT TTG AAA TAA TAT TG which included a *Bam*H I site downstream of the stop codon; primers were made using an Applied Biosystems 391 DNA Synthesiser. PCR was conducted using a cycle program of: 1 cycle of 94°C for 1 minute; 30 cycles of: 94°C for 1 minute, then 52°C for 2 minutes, and 72°C for 3 minutes; and finally 1 cycle of 72°C for 7 minutes using an automatic cycler (Omnigene; Hybaid). The reaction buffer contained 300 mM Tris-HCl, 75 mM (NH₄)₂SO₄, and 10 mM MgCl₂, at pH 8.5 (at 22°C). Primers were added to 0.05 µg / µl; dNTPs (Pharmacia) were added to a final concentration of 1 mM; Taq polymerase (Pharmacia) at 1 unit / 50 µl of reaction; and sample DNA at 1 µl / 50 µl of reaction.

The two oligonucleotide primers that flank the DNA fragment to be amplified hybridise to opposite strands of the target sequence and direct DNA synthesis by the polymerase across the region between the primers. Since the extension products themselves are also complementary to and capable of binding primers, successive cycles of amplification double the amount of the target DNA synthesised in the previous cycle to give an exponential accumulation of the specific target fragment.

7.4.9 Cloning of the putative enterotoxin gene into plasmid pTrc 99A

Plasmid pTrc 99A is a derivative of the expression vector pKK233-2, with a strong promoter (*Trc*) upstream of a multiple cloning site (MCS) and a strong transcription termination signal (*rrnB*) downstream. It contains the *lac I^q* gene which allows it to be used in *E. coli* hosts lacking the lactose repressor gene. With an expanded MCS, the vector can accept inserts having a variety of endonuclease-generated ends. An *Nco* I restriction site is juxtaposed next to the *Trc* promoter so that inserts possessing the translational start codon ATG can be ligated and directly expressed. DNA potentially encoding the enterotoxin gene obtained by polymerase chain reaction was designed with the objective of ligating the start codon of the enterotoxin gene into the *Nco* I site and the stop codon five bases upstream from the *Bam*H I site of the expression plasmid pTrc 99A (Pharmacia). PCR fragments were purified using Magic DNA Clean-up System (Promega).

The plasmid was isolated from transformed *E. coli* JM105 using a Qiagen plasmid midi extraction kit (Qiagen Inc.). The plasmid and PCR product DNA was cleaved with *Bam*H I and *Nco* I (Pharmacia) according to the suppliers instructions and buffer systems. This generated complementary single stranded extensions which would direct insertion of the enterotoxin gene in the correct orientation. The plasmid DNA and PCR fragments then associated by means of base paired ends which were ligated by 1 unit of T4 DNA ligase (Pharmacia) which formed a phosphodiester linkage between the termini of the DNA fragments, linking them covalently. The suppliers buffer system supplemented with 1 mM ATP (final concentration; Pharmacia) was used. An insert : vector ratio (molar or DNA concentration) of

1 : 2.5 was used in the ligation mixture with a total DNA content of 26 ng in a total volume of 10 μ l.

One ml of an overnight culture of *E. coli* JM105 was subcultured into 100 ml of LB broth (Sigma) and incubated at 37°C shaking at 200 rpm (revolutions per minute) for 3.5 hours. The culture was centrifuged at 10,000 g, at 4°C, for 5 minutes and the supernatant fraction drained. The JM105 *E. coli* cells were resuspended in 25 ml of chilled 0.1 M calcium chloride, and incubated on ice for 15 minutes. The cells were centrifuged at 10,000g, at 4°C. for 5 minutes and the supernatant drained, the cells are then finally resuspended in 4 ml 0.1 M calcium carbonate. 200 μ l of the competent cells are then incubated with 10 μ l of a 1 / 10 dilution of the vector : insert ligation mixture (9 ng of DNA / μ l) in LB broth (see section 7.4.3), at 42°C for 2 minutes. Then 1 ml of LB broth (Sigma) was added and incubated at 37°C for 1 hour to allow the cells to take up the circular DNA. 100 μ l of the undiluted and two 10-fold dilutions of the suspension were plated onto LB plates with 0.01% (w / v) ampicillin and incubated at 37°C aerobically overnight.

Cells which acquired the vector DNA or the vector DNA with the enterotoxin gene were selected for by ampicillin resistance.

7.4.10 Detection of recombinant pTrc 99A

There were approximately 11 colonies per plate inoculated with *E. coli* transformed with the ligation reaction containing PCR product; plates inoculated with *E. coli* transformed with the ligation reaction containing the plasmid alone, had less than 1% of this number of colonies.

Ten colonies of *E. coli* JM105 transformed with the ligation reaction containing the PCR product were cultured in LB broth (Sigma) with 0.01 % (w / v) ampicillin, for 18 hours at 37°C with shaking at 200 rpm. The cultures were centrifuged at 12,000 g for 30 seconds and the cell pellets were resuspended in 100 μ l of 25 mM Tris, 50 mM glucose, 10 mM EDTA with 0.5 % (w / v) lysozyme and incubated at 0°C for 15 minutes. 200 μ l of alkaline-SDS solution (0.2 N NaOH, 1% (w / v) SDS) was then added to the cells which were kept at 0°C for a further

5 minutes. 150 µl of a high salt solution (3M sodium acetate pH4.8) was added and gently mixed, then kept at 0°C for at least 60 minutes. The precipitate was spun down at 10,000 g at 4°C for 10 minutes. DNA in the sample fraction was then purified by the Magic DNA Clean-up System (Promega) used according to the manufacturers instructions.

6 µl of purified plasmid was digested in 1 x One Phor All Plus buffer (Pharmacia) in a total of 5 µl with 0.3 units of *Nco* I and 0.15 units of *Bam*H I restriction enzymes (Pharmacia) at 37°C for 1 hour. Digests (5 µl) were mixed with 5 µl of gel loading solution (Sigma) and analysed by agarose gel electrophoresis (see section 7.4.6). Those plasmids containing an insert of approximately 1 Kb were presumptively considered to be potential enterotoxin gene recombinants which were studied further; by induction of enterotoxin gene expression (see section 7.4.11) and sequencing of the fragment following sub-cloning into M13 (see section 7.4.12).

7.4.11 Induction of enterotoxin gene expression

The enterotoxin gene recombinant p*Trc* 99A plasmids in JM105 *E. coli* were cultured overnight in LB broth with 0.01 % (w / v) ampicillin in a shaking incubator at 37°C. The following day the culture was diluted 1 / 20 in LB broth with 0.01 % (w / v) ampicillin and incubated with shaking at 37°C for 2 hours. Finally, IPTG (isopropyl-β-thiogalactopyranoside) was added to 2 ml of the culture to a final concentration of 5 mM and sub-samples were taken after 2, 4 and 6 hours incubation at 37°C. These were mixed with an equal volume of SDS-PAGE sample buffer (see section 7.4.7), boiled for 2 minutes, centrifuged at 12,000 g for 5 minutes and the supernatant fractions were analysed by SDS-PAGE gels which were silver stained (see section 7.4.7 and Figure 5). Additional sub-samples were taken at the same time intervals and the production of enterotoxin was detected by RPLA and cytotoxicity assays (see section 7.4.1). Control cultures of *E. coli* JM105 containing parent p*Trc* 99A were treated in the same manner.

7.4.12 Single stranded DNA sequencing

The enterotoxin gene was transferred from p*Trc* 99A to the M13mp19 single

stranded DNA bacteriophage to aid its sequencing. This phage has an intermediate replicative double stranded form which enables it to be handled like a plasmid particularly with respect to cloning and *E. coli* transfection.

A stock of M13mp19 DNA was prepared by transfecting *E. coli* TG1 with double stranded replicative form (ds rf) of the phage. TG1 was grown on M9 minimal agar (see section 7.4.3) to promote F' episomes, which are required for transfection (the F pili encoded by the episome are the receptors for this phage) during the stages of phage propagation. The TG1 plating bacteria were prepared by overnight culture of one colony in 50 ml SOB medium (see section 7.4.3) at 37°C with constant shaking at 200 rpm. Competent cells of TG1 were prepared by the calcium chloride method (see section 7.4.9) and 1 µl of ds rf DNA M13mp19 was added to 50 µl of the cells, then chilled on ice for 30 minutes. Meanwhile tubes of molten SOB agar (see section 7.4.3) were prepared and stored in a water bath at 47°C. The cells were then removed from the ice and incubated at 42°C for 90 seconds, and then returned to the ice bath. After 2 minutes 175 µl of SOB medium (see section 7.4.3) lacking magnesium was added. 40 µl XGal (20 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside / ml in dimethyl formamide; Sigma) and 4 µl IPTG (200 mg isopropyl-β-thiogalactopyranoside / ml; Sigma) were added to the molten SOB agar and mixed. Aliquots of 1, 10, and 100 µl of transfected bacteria were added to a series of tubes and 200 µl of competent cells were then added to each of the tubes. The tubes were mixed and poured onto LB plates, allowed to set and then incubated aerobically, for 12 hours, at 37°C. Three discrete blue plaques were then picked with a wooden applicator stick and placed in 3 ml of LB broth (see section 7.4.3), left to stand at room temperature and then 2.5 ml was placed in 50 ml of LB broth (see section 7.4.3) with 2.5 ml of plating bacteria, incubated at 37°C for 6 hours with constant shaking at 200 rpm, then centrifuged at 4°C, and 5000 g for 15 minutes and the ds rf DNA was extracted using a Qiagen midi column (Qiagen) according to the manufacturers instructions.

Three of the putative enterotoxin gene *pTrc* 99A recombinants were selected

for sequencing and their plasmids were purified as described above (see section 7.4.9).

The M13mp19 extracts (50 μ l) and p*Trc* 99A enterotoxin gene recombinants extracts (50 μ l) were digested with 0.3 units of *Hinc* II and 0.15 units of *Bam*H I at 37°C for 1 hour then heat killed at 85°C for 30 minutes. The target fragment in the p*Trc* 99A enterotoxin gene recombinants was separated by electrophoresis (see section 7.4.6) in low melting point agarose without ethidium bromide. A small slice of the gel including the molecular weight marker and a section of gel containing digested plasmid was stained with ethidium bromide after electrophoresis. The stained section was viewed on an ultra-violet transilluminator and the position of the required 1 Kb fragment marked, the stained gel was re-aligned with the unstained section and the target fragment was cut out, melted and then recovered using a Magic DNA clean-up column (Promega) according to the manufacturers instructions.

The M13mp19 DNA (0.21 μ g in 50 μ l) was dephosphorylated with 1 unit of alkaline phosphatase (Pharmacia) for 30 minutes before stopping the reaction at 85°C for 15 minutes according to the manufacturers instructions, to force a directed ligation, followed by DNA Magic clean-up (Promega) used according to the manufacturers instructions. The enterotoxin gene fragment was then ligated into M13mp19 (see section 7.4.9) using a vector / insert ratio of 1 : 3 (total DNA of 26 ng / reaction in 10 μ l with 0.2 units of ligase (Pharmacia) at room temperature overnight, using the suppliers buffer system supplemented with 1 mM ATP (final concentration; Pharmacia). Three sets of reactions were performed, one for each of the original p*Trc* 99A recombinants.

Selection of the recombinant bacteriophages depended on the insertion of additional DNA into the polycloning site of M13mp19 which destroyed α -complementation of the β -galactosidase activity and created recombinant bacteriophages that produced colourless plaques when grown in the presence of IPTG and XGal (a chromogenic substrate for β -galactosidase), instead of the non-recombinant blue plaques. IPTG induced β -galactosidase activity as a lactose

analogue by binding and inhibiting the lac repressor. The colourless plaques were picked, replicated and ds rf DNA prepared as above. The extracts (20 μ l) were digested with 0.3 units of *Hinc* II using the conditions described for the double digest above and then run on an agarose electrophoresis gel (see section 7.4.6). One recombinant with a DNA band approximately 1 Kb larger than the non-recombinant strains was chosen from each batch of ligation reactions, so that each of the three original p*Trc* 99A recombinants could be sequenced.

Single stranded recombinant M13mp19 DNA was prepared from phage particles for the sequencing reactions. Following replication of the recombinants (as above), 1.5 ml of the supernatant fraction was taken and 210 μ l of 20% (w / v) polyethylene glycol (PEG) 6000 (Sigma) in 2.5 M sodium chloride solution was added, the tube inverted and then left at room temperature for 15 minutes. The tube was then centrifuged at 12,000g for 5 minutes and the supernatant fraction discarded; the centrifugation was repeated and the PEG removed. 100 μ l of TE buffer (10 mM Tris-HCl pH 7.5 with 1 mM EDTA) and 100 μ l of chloroform were added and the mixture was vortexed for 20 seconds and centrifuged at 12,000 g for 3 minutes. The supernatant fraction was recovered to which 50 μ l of buffered phenol, pH 8, was added; the mixture was vortexed for 20 seconds, left at room temperature for 15 minutes, vortexed for 20 seconds and centrifuged at 12,000 g for 3 minutes. 500 μ l of ether was added to the supernatant fraction, vortexed, centrifuged at 12,000 g for 1 minute and the ether layer removed. The remaining ether was removed by heating at 65°C until the bubbling stopped. 10 μ l of 3 M sodium acetate and 250 μ l of ice cold absolute alcohol was added and the tube left at minus 20°C overnight. The tube was then centrifuged at 12,000 g and 4°C, for 10 minutes, washed with 1 ml of 70% (v / v) ice cold ethanol, and recentrifuged for 5 minutes. The supernatant fraction was removed and the pellet was allowed to dry, before it was resuspended in 28 μ l of TE buffer.

The Sanger dideoxynucleotide chain termination method (Sanger *et al.*, 1977) for single stranded sequencing of deoxyribonucleic acid was used (Sequenase,

United States Biochemical) according to the kit manufacturers instructions. 1 µg of DNA (7 µl of the ssDNA preparations prepared as above) was used in the sequencing reactions.

Briefly, short complementary primers to the phage ssDNA provided a target for complementary chain extension by polymerase. 1 µg of the DNA to be sequenced was annealed with 0.05 pmol of primer sequence (5'-GTTTTCCCAGTCACGAC-3') in 40 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, and 50 mM NaCl, by heating for 2 minutes at 65°C and cooling slowly to less than 35°C over 15 to 30 minutes. Meanwhile the termination reactions (one for each base) were prepared and warmed to 37°C. The four termination mixtures consisted of 80 µM each of dGTP, dATP, dCTP, and dTTP, and 50 mM NaCl, with 8 µM of one of the dideoxynucleotides for each of the reactions. The labelling reaction consisted of 10 µl of the cooled annealed DNA reaction (see above), 6 mM dithiothreitol, 1.5 µM dGTP, 1.5 µM dCTP, 1.5 µM dTTP, 10 µM [α -³²P] dATP, 3.25 µl Sequenase polymerase which was incubated at room temperature for 5 minutes. 3.5 µl of the labelling reaction was added to each of the four termination mixtures (see above), and incubated at 37°C for 5 minutes. The reactions were stopped by the addition of 38 % (v / v) formamide, 8 mM EDTA, 0.02 % (w / v) Bromophenol Blue, 0.02 % (w / v) Xylene Cyanol FF (final concentrations). The reactions were heated to 75°C 2 minutes before loading 2 µl onto a sequencing gel. The relative concentrations of ddNTPs and dNTPs allowed random incorporation of a ddNTP at the relevant position for a considerable length of synthesised DNA. Each ddNTP prevented further synthesis of that strand so that finally each reaction contained a mixture of DNA of different lengths corresponding to the addition of a ddNTP. These were separated by PAGE and their location detected by autoradiography of the incorporated [α -³²P] dATP. Comparison of the migration of the DNA strands in each reaction, one for each ddNTP, provided the sequence.

Sequencing gels were prepared in electrophoresis gel apparatus designed for sequencing (Bio-Rad), according to the manufacturers instructions. A 6%

polyacrylamide gel was prepared (as section 7.4.7) including 7 M urea. The buffer reservoirs were filled with 1 x TBE buffer (89 mM Tris borate pH 8.3 with 2.5 mM EDTA) and the gel prewarmed by applying power of 1700 V, 70 W constant power for about 30 minutes, before loading sequencing samples. The preformed wells were rinsed with 1 x TBE before the samples were loaded, which were preheated to 95°C for 2 minutes. The gels were run at a constant power of 70 W, to maintain the gel temperature of 65°C for between 2 to 5 hours depending on the progress of the marker dyes and the area of interest of the sequence.

The gels were removed from the apparatus on Whatman 3MM paper, covered with polyvinyl chloride film and dried in commercial gel drying apparatus (Bio-Rad). The film was removed from the dried gel, placed in a light-tight cassette, an X-ray film (Fuji Rx; Genetic Research International) was placed over it and the loaded cassette kept at room temperature for approximately 3 days. The films were then processed using an automatic developer according to the manufacturers instructions (Curix 60; Agfa-Gevaert Ltd.).

Complementary primers were made (Applied Biosystems 391 DNA Synthesiser) upstream of the 3' end of the preceding section of sequence generated (the first obtained by using the Sequenase kit M13 primers), and were used to continue sequencing. The sequence was input by digitizer into the DNASIS program (Hitachi Software Engineering Co., Ltd., 1991) and the different sections of sequence were analysed for overlapping homology and combined. The sequences of the three M13 clones were compared with each other and the previously published sequences for the enterotoxin gene.

7.4.13 Statistical Analyses

Data were analysed by multivariate analysis of logistic regression models as described in Chapter Three using the model without *S. westeri* to conserve processing power. This was considered acceptable because inclusion of *S. westeri* whilst greatly increasing the need for processing power did not significantly affect the fit of the multivariate model for the other significantly associated pathogens.

7.5 RESULTS AND DISCUSSION

Direct detection of enterotoxin in faeces by RPLA was significantly associated with diarrhoea as was detection of enterotoxin production from isolates (Table 22). The strain B3550.5 isolated from a foal with diarrhoea produced large amounts of an antigen that reacted in the RPLA test and its culture supernatant fractions were toxic for Vero cells; more than 75% of cytotoxicity could be neutralised by antiserum to enterotoxin. See Addendum 1 (p151)

PCR using DNA extracted from strain B3550.5 and primers derived from the enterotoxin gene sequence of a human isolate yielded a fragment of approximately 1 Kb expected of the enterotoxin gene, a similar fragment was seen after PCR of DNA extracted from the enterotoxigenic *C. perfringens* strain NCTC8239 (see Figure 4). The PCR product from B3550.5 DNA when cloned into pTrc 99A gave several recombinants of which 3 were characterised further. Each recombinant was toxigenic by the RPLA test, verotoxigenic and yielded a 1 Kb fragment after digestion with the restriction enzymes *Nco* 1 and *Bam*H 1 similar to that produced by PCR of B3550.5 or NCTC8239 DNA; *E.coli* JM105 with pTrc 99A (non-recombinant) was negative in all of these tests. Titration of overnight culture supernatants of strain B3550.5 sporulated in Duncan and Strong medium, and of the recombinant *E.coli* grown in LB broth, by both RPLA and Vero-cell toxicity tests, showed that the recombinant *E.coli* produced 10-fold more reactive component than strain B3550.5. Production of the putative enterotoxin was constitutive without lacZ induction. Induction of the gene with IPTG increased the amount of assayable toxin in culture supernatant fractions 10-fold. Analysis of the *E. coli* JM105 recombinant and non-recombinant culture supernatant fractions by SDS-PAGE revealed an extra 35kDa protein fragment in extracts of the recombinant (see Figure 5), which was the same size as previously published for the native enterotoxin molecule (Van Damme-Jongsten *et al.*, 1989). The molecular weight predicted from the published sequence is 35,391 (Czeczulin *et al.*, 1993).

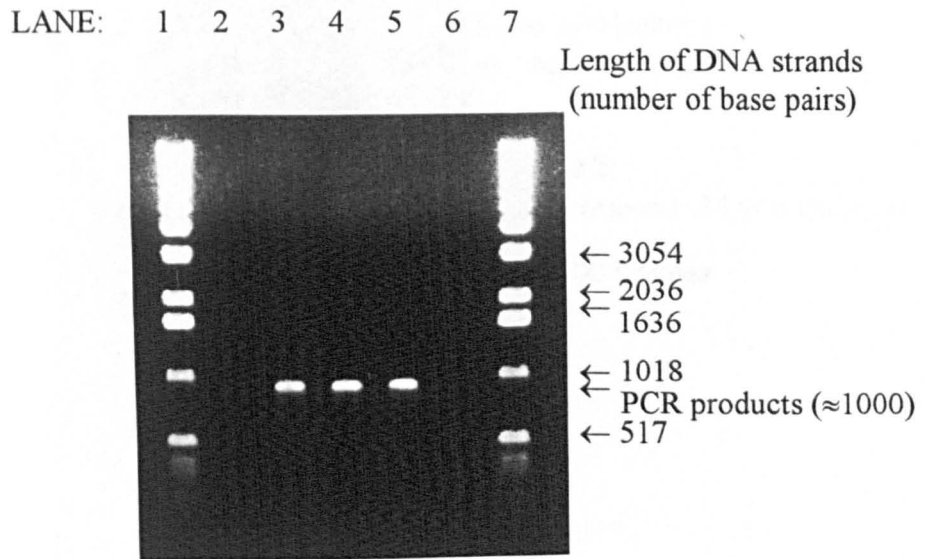
The inserts from the plasmids of the three recombinants were separately

subcloned into bacteriophage M13mp19 and each was separately sequenced (see Figure 6). All of the subcloned fragments had the same sequence with the exception of base 894, where in two of the clones a cytosine was substituted for a thymidine residue present in the previously published DNA sequence. This substitution did not alter the primary sequence. This variation was probably caused by a polymerase reading error. The only difference in sequence with that published for the human isolate, common to all the subclones, was in the first position of the second codon (position 4). Since this was introduced intentionally to facilitate cloning into p*Trc* 99A it is likely, in view of the extensive homology between the rest of the sequences, that the native gene would have had the same codon as that published for the human isolate. This substitution resulted in a conservative change of amino acid from leucine to valine.

Enterotoxigenic *C. perfringens* isolates were significantly associated with diarrhoea (OR = 6.7; $p < 0.001$; see Table 22) although only a small proportion of foals with diarrhoea were positive (24%). The strength of association with diarrhoea, however, was almost as large as for all *C. perfringens* combined (Table 13, Chapter Three). The presence of enterotoxin in the faeces samples was also significantly associated with diarrhoea in foals (OR = 3.1; $p = 0.009$), although fewer samples were RPLA positive than yielded enterotoxigenic isolates by culture.

Although only 24% of foals were positive for enterotoxigenic *C. perfringens* isolates, these represented 42% of scouring foals which were positive for *C. perfringens*. This suggested that the RPLA may be insensitive as a method of enterotoxin detection and that the true rate of enterotoxigenicity amongst isolates from scouring foals may be greater. The confirmation of the carriage of the enterotoxin gene identical to that described for a human isolate and its cloning by PCR reaction suggested the possibility of testing DNA extracted from isolates for the gene. The use of PCR to estimate the prevalence of enterotoxin gene possession in the foal isolates of *C. perfringens* is described in Chapter Five.

FIGURE 4 Photograph of an agarose gel with PCR products of DNA extracts of *Clostridium perfringens* B3550.5, NCTC8239, and *E. coli* JM105 pTrc 99A recombinant containing the enterotoxin gene.



KEY :

LANE:

1 4 µl of 1 Kb DNA Ladder (GIBCO BRL)

2 Blank

3 PCR product of *C. perfringens* B3550.5 DNA extract*

4 PCR product of *C. perfringens* NCTC8239 DNA extract*

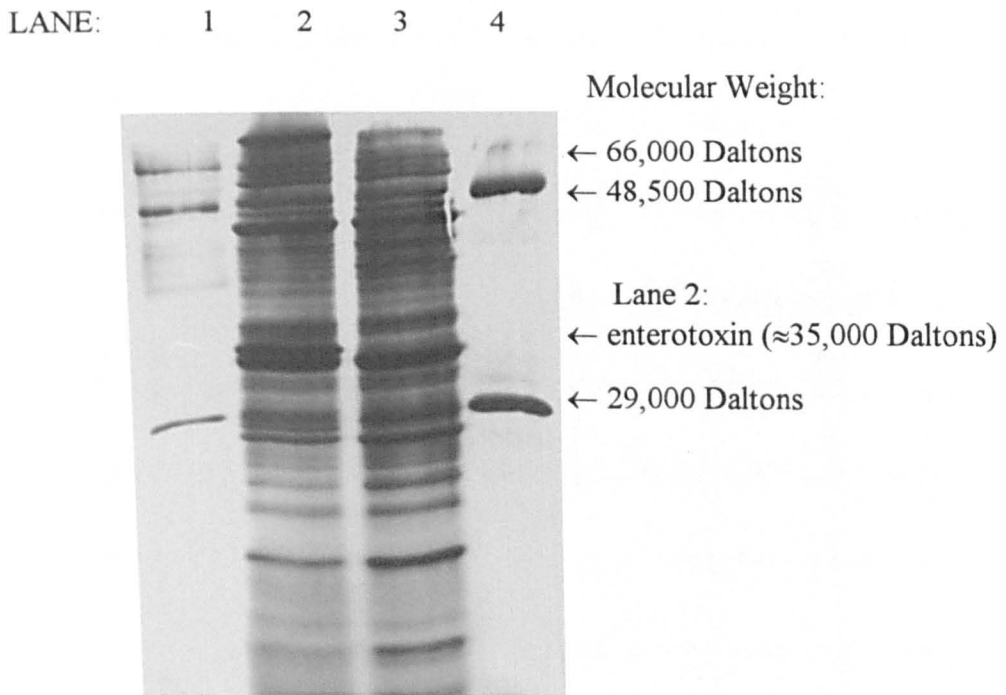
5 PCR product of *E.coli* JM105 recombinant with the enterotoxin gene DNA extract*

6 Blank

7 4 µl of 1 Kb DNA Ladder (GIBCO BRL)

*4 µl of each PCR product was mixed with 4 µl of gel loading solution (Sigma) before running the gel (see section 7.4.6).

FIGURE 5 Photograph of an SDS-PAGE gel of the *E. coli* JM105 p*Trc* 99A recombinant and non-recombinant culture supernatant fractions.



KEY

LANE:

- 1 High range silver stain SDS molecular weight standard mixtures (Sigma).
- 2 Culture supernatant fraction of *E. coli* JM105 with p*Trc* 99A enterotoxin gene recombinant (see section 7.4.11 and 7.4.7).
- 3 Culture supernatant fraction of *E. coli* JM105 with the parent p*Trc* 99A (see section 7.4.11 and 7.4.7)
- 4 Low range silver stain SDS molecular weight standard mixtures (Sigma).

FIGURE 6 Sequence of the *Clostridium perfringens* B3550.5 enterotoxin gene.

1	11	21	31	41	51
ATGGT ^{C*} TAGTA	ACAATTTAAA	TCCAATGGTG	TTCGAAAATG	CTAAAGAAGT	ATTTCTTATT
61	71	81	91	101	111
TCTGAGGATT	TAAAAACACC	AATTAATATT	ACAAACTCTA	ACTCAAATTT	AAGTGATGGA
121	131	141	151	161	171
TTATATGTAA	TAGATAAAGG	AGATGGTTGG	ATATTAGGGG	AACCCTCAGT	AGTTTCAAGT
181	191	201	211	221	231
CAAATTCTTA	ATCCTAATGA	AACAGGTACC	TTTAGCCAAT	CATTAECTAA	ATCTAAAGAA
241	251	261	271	281	291
GTATCTATAA	ATGTAAATTT	TTCAGTTGGA	TTTACTTCTG	AATTTATACA	AGCATCTGTA
301	311	321	331	341	351
GAATATGGAT	TTGGAATAAC	TATAGGAGAA	CAAAATACAA	TAGAAAGATC	TGTATCTACA
361	371	381	391	401	411
ACTGCTGGTC	CAATGAATA	TGTATATTAT	AAGGTTTATG	CAACTTATAG	AAAGTATCAA
421	431	441	451	461	471
GCTATTAGAA	TTTCTCATGG	TAATATCTCT	GATGATGGAT	CAATTTATAA	ATTAACAGGA
481	491	501	511	521	531
ATATGGCTTA	GTAAACATC	TGCAGATAGC	TTAGGAAATA	TTGATCAAGG	TTCATTAATT
541	551	561	571	581	591
GAAACTGGTG	AAAGATGTGT	TTTAACAGTT	CCATCTACAG	ATATAGAAAA	AGAAATCCTT
601	611	621	631	641	651
GATTTAGCTG	CTGCTACAGA	AAGATTAAAT	TTAACTGATG	CATTAACTC	AAATCCAGCT
661	671	681	691	701	711
GGTAATTTAT	ATGATTGGCG	TTCTTCTAAC	TCATACCCTT	GGACTCAAAA	GCTTAATTTA
721	731	741	751	761	771
CACTTAACAA	TTACAGCTAC	TGGACAAAAA	TATAGAATCT	TAGCTAGCAA	AATTGTTGAT
781	791	801	811	821	831
TTTAATATTT	ATTCAAATAA	TTTTAATAAT	CTAGTGAAAT	TAGAACAGTC	CTTAGGTGAT
841	851	861	871	881	891
GGAGTAAAAG	ATCATTATGT	TGATATAAGC	TTAGATGCTG	GACAATATGT	TCTCGTAATG
901	911	921	931	941	951
AAAGCTAATT	CATCATATAG	TGGAAATTAC	CCTTATTCAA	TATTATTTCA	AAAATTTTAA

* G was substituted for C in the PCR primer to create an *Nco* I site for cloning into pTrc 99A.

** T was present in one subclone (as in the previously published sequence), however C was present in the 2 other subclones.

8.0 CHAPTER FIVE

DETECTION OF THE *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN GENE IN EQUINE ISOLATES AND ITS ASSOCIATION WITH FOAL DIARRHOEA

8.1 CONTENTS

8.1.1 List of Tables	103
8.1.2 List of Figures	103
8.2 Introduction	104
8.3 Objectives	105
8.4 Methods and Materials	106
8.4.1 Polymerase chain reaction	106
8.4.2 Enterotoxin gene probe	106
8.4.3 Statistical Analyses	107
8.5 Results and Discussion	108

8.1.1 List of Tables

Table 23. Detection of the enterotoxin gene by Polymerase Chain Reaction from <i>C. perfringens</i>	110
Table 24. Reaction of isolates positive or negative by RPLA in the PCR, by a gene probe against chromosomal DNA extract, by a gene probe against PCR product and by a test of verotoxicity neutralised by anti-enterotoxin serum	111
Table 25. Univariate analysis of the numbers of <i>C. perfringens</i> cfu's per gram of faeces isolated from foals not-in-contact with cases of diarrhoea	112
Table 26. Univariate analysis of the association of PCR positive <i>C. perfringens</i> with large numbers of cfu's per gram of faeces ($\geq 10^4$) isolated by three different methods	112

8.1.2 List of Figures

FIGURE 7. Distribution of the number of enterotoxigenic <i>C. perfringens</i> identified by RPLA and PCR methods	110
---	-----

8.2 INTRODUCTION

It has been established that the isolation of *C. perfringens* (OR = 7.4; $p < 0.001$), the isolation of enterotoxigenic *C. perfringens* (OR = 6.7; $p < 0.001$), and the detection of enterotoxin directly in the faeces (OR = 3.1; $p < 0.009$) are significantly associated with foal diarrhoea (see Chapters Three and Four). Enterotoxigenic *C. perfringens* represented only a fraction of the *C. perfringens* isolates overall but their strength of association with diarrhoea was similar to that of undifferentiated *C. perfringens*. If the enterotoxin was the only virulence mechanism enabling *C. perfringens* to cause diarrhoea it would be expected that the strength of association of enterotoxigenic *C. perfringens* would be greater than for all *C. perfringens* combined. The fact that the strength of association was not greater suggested that either enterotoxin production was just one of the mechanisms involved in the pathogenesis of *C. perfringens* foal diarrhoea or that the method of detection underestimated the number of *C. perfringens* which were enterotoxigenic, or that there was no causal relationship.

The sequence of the enterotoxin gene from an equine isolate was virtually identical to that of human isolates (see Chapter Four), thus PCR amplification could be used to detect the presence of the gene. Testing the *C. perfringens* isolates from foals for the presence of the enterotoxin gene, by PCR, would help to clarify the accuracy of the RPLA method in detecting enterotoxigenic *C. perfringens* and in particular assess if it seriously underestimated the prevalence of potential enterotoxigenicity in the survey isolates.

PCR methods and probes based on partial sequences of the enterotoxin gene, previously have been used to test *C. perfringens* isolates. A survey of randomly chosen isolates from farm animals suggested that 6% contained the enterotoxin gene (Van Damme-Jongsten *et al.*, 1989), while 59% of isolates from food and faeces, associated with food poisoning outbreaks of known *C. perfringens* aetiology in man contained the gene (Van Damme-Jongsten *et al.*, 1990a). A screen of porcine isolates, however did not detect the enterotoxin gene in association with disease (Van Damme-Jongsten *et al.*, 1990b).

8.3 OBJECTIVES

The objectives were:

1. To develop molecular genetic methods for the detection of the enterotoxin gene in isolates from the survey of foal diarrhoea.
2. To compare the results of these methods with the results of RPLA.
3. To assess the prevalence of the enterotoxin gene in equine isolates.
4. To test if there is any association between possession of the enterotoxin gene by isolates of equine *C. perfringens* and foal diarrhoea.

8.4 METHODS AND MATERIALS

8.4.1 Polymerase chain reaction

The enterotoxin gene of *C. perfringens* isolates from foal diarrhoea was amplified by PCR (see Chapter Four section 7.4.8). The reaction was optimised for testing of all of the *C. perfringens* isolates by changes in buffer components (PCR Optimizer; Invitrogen Corporation) and by reduction of the amount of target DNA. The three main stages of the process, PCR preparation, amplification and product analysis, were carried out in different laboratories with equipment dedicated to each stage of the process to eliminate cross contamination of PCR product and sample DNA. PCR reactions were analysed by agarose gel electrophoresis for a fragment of the expected size for the enterotoxin gene (see section 7.4.1; Chapter Four).

8.4.2 Enterotoxin gene probe

The PCR product from the cloned enterotoxin gene was isolated by gel electrophoresis (see Chapter Four section 7.4.6) and labelled by the random primer method (Sambrook *et al.*, 1989), where the target sequence was used as a template for the enzymatic construction of a complementary strand which included ^{32}P radiolabelled dATP in the dNTP mixture.

The radio-labelled probe was hybridised with the test sample DNA which was immobilised on Hybond N membrane (Amersham International Limited) by the dot blot method (Sambrook *et al.*, 1989). The sample DNA was denatured before application to the membrane and hybridisation to the gene probe. *C. perfringens* chromosomal DNA was prepared as in section 7.4.4; Chapter Four.

Different denaturing methods were used for chromosomal DNA samples and the PCR products, before application to the membrane. The chromosomal DNA was denatured by heating at 95°C for 5 minutes, whilst the PCR product was denatured by heating to 37°C for 10 minutes in 0.5 M sodium hydroxide before application to the membrane. The samples were allowed to dry on the membrane and were then further denatured with 1.5 M NaCl, 0.5 M NaOH solution, neutralised with 1.5 M

NaCl, 0.5 M Tris-HCl, pH 7.2, 0.001 M Na₂ EDTA solution and allowed to dry again. The DNA was fixed to the membrane by UV irradiation for 5 minutes.

The membrane was then pretreated for at least one hour at 65°C with a pre-hybridisation buffer [3 x SSC (Sigma), 100 µl 1 x Denhardt's buffer, 100 µl 1% (w / v) Herring sperm DNA and milliQ water to 10 ml; Denhardt's buffer consists of 1g ficoll 400 (Pharmacia), 1g polyvin pyrrolidine MW 40,000 (Sigma) and milliQ water (Millipore) to 50 ml] to block the areas of the membrane that have not bound DNA and so prevent non-specific binding of the probe. The labelled enterotoxin gene probe was added to the pre-hybridisation buffer, and the hybridisation of the sample DNA with the labelled probe was continued overnight. This was followed by successive washes in solutions of increasingly lower salt concentrations and higher SDS concentrations to remove unbound probe. The first wash was 2 x SSC (Sigma) with 0.01% (w / v) SDS at 65°C for 30 minutes, then repeated twice, and followed by a wash of 0.1 x SSC with 0.1% (w / v) SDS. The membranes were then dried before autoradiography. The dry membranes are wrapped in polyvinyl chloride film, placed in a light-tight cassette, an X-ray film (Fuji Rx; Genetic Research International) placed over it, followed by an intensifying screen, and the loaded cassette kept at minus 70°C, for three days. The films were then processed using an automatic developer according to the manufacturers instructions (Curix 60; Agfa-Gevaert Ltd.).

The sample DNA dots with darkened areas, produced by the action of the radioactivity on the X-ray film, were then recorded as positive for the possession of the enterotoxin gene.

8.4.3 Statistical Analyses

As with methods used in Chapter Four a model without *S. westeri* was used. Furthermore, because the enterotoxin gene was not detected in any *C. perfringens* isolates from healthy not in contact foals, all healthy foals were used as the control group.

8.5 RESULTS AND DISCUSSION

The Polymerase chain reaction using conditions employed for cloning the enterotoxin gene and DNA extracted from 8 strains positive by RPLA resulted in only one strain giving a product of the expected size in agarose gel electrophoresis. Attempts to optimise the PCR by alteration of the pH or Mg^{++} ions did not increase the number of positive isolates. Dilution of the chromosomal DNA extracts by 100-fold, however, resulted in a further two isolates as well as the isolate originally positive by PCR yielding a fragment in agarose gel electrophoresis of the expected size for the enterotoxin gene.

The isolates from the survey of foal diarrhoea were analysed by PCR and gel electrophoresis of the product. Although far fewer isolates reacted in the PCR test than by the RPLA test (Chapter Four; section 7.4.1) possession of the enterotoxin gene was still associated with foal diarrhoea (Table 23; odds ratio, 19.1; $p < 0.005$); none of the isolates from cases of diarrhoea with a fatal outcome produced evidence of the enterotoxin gene. Twelve of the PCR positive isolates were negative by RPLA (see Figure 7) which was possibly caused by poor sporulation and, therefore, inadequate enterotoxin production which is needed for this test to work.

The discrepancy between the RPLA and PCR tests prompted a more detailed analysis of the relationship between them. A representative number of isolates from separate cases of diarrhoea and from healthy foals were compared with the control enterotoxigenic *C. perfringens* NCTC 8239 by RPLA, PCR and reaction of the labelled cloned gene with chromosomal DNA and PCR product (Table 24). None of the six RPLA negative isolates tested from healthy foals reacted in any of the genetic tests for the enterotoxin gene. All of the isolates positive by one of the genetic tests (PCR, gene probe of DNA extract, gene probe of PCR product) were positive by all of the other genetic tests. All isolates showed some signs of toxicity in the vero cell assay but in only three was it noticeably reduced by antiserum to the enterotoxin and these were all positive by

the genetic tests for the enterotoxin gene. Five of the isolates positive by RPLA did not react in any of the genetic tests for the enterotoxin gene and none of these produced verotoxicity which could be neutralised by antiserum to enterotoxin.

Tests did not reveal an association between isolation of a PCR positive *C. perfringens* and the presence of greater than 10^4 *C. perfringens* cfu per gram of faeces for direct plate culture or heat treatment (Tables 25 and 26). However, an association was found between the presence of greater than 10^4 cfu per gram of faeces and the isolation of a PCR positive *C. perfringens* by alcohol treatment (Tables 25 and 26). Failure to detect an association by the other isolation and enumeration methods probably relates to the small number of samples with large numbers of *C. perfringens* and the small number of *C. perfringens* that were enterotoxigenic.

Two of the four faeces samples with frank blood (Chapter Three) were from post mortem cases positive for *C. perfringens*. As has already been noted, none of the *C. perfringens* from cases with a fatal outcome were positive for the enterotoxin gene. However, enterotoxigenic *C. perfringens* were isolated from the two other samples containing blood.

Enterotoxigenic isolates of *C. perfringens* from eight out of eleven scouring foals were not serotypable. The other three yielded enterotoxigenic isolates belonged to serotypes 51, 7, TW5 and 8 (serotyping carried out at the Food Hygiene Laboratory, Colindale).

See Addendum 2 (p152)

Table 23 Detection of the enterotoxin gene by Polymerase Chain Reaction from <i>C. perfringens</i> (number of foals tested).				
Detection method	Percentage positive faeces from foals with diarrhoea (n = 421)	Percentage positive faeces from all healthy foals (n = 222)	Percentage positive faeces from healthy foals in-contact with diarrhoea cases (n = 102)	Percentage positive faeces from healthy foals not-in-contact with diarrhoea cases (n = 120)
Enterotoxigenic <i>C. perfringens</i> (RPLA)	24	8	8	8
Enterotoxin in faeces (RPLA)	9	4	8	1
<i>C. perfringens</i> enterotoxin gene detection by PCR	5	0.5	1	0

FIGURE 7. Distribution of the number of enterotoxigenic *C. perfringens* identified by RPLA and PCR methods (n = 421).

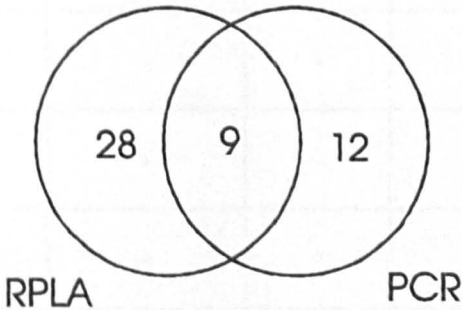


Table 24 Reaction of isolates positive or negative by RPLA in the PCR, by a gene probe against chromosomal DNA extract, by a gene probe against PCR product and by a test of verotoxicity neutralised by anti-enterotoxin.

Isolates From separate cases of diarrhoea	RPLA (repeat result)	Probe of DNA extracts	PCR	Probe of PCR product	Vero toxic ^a
1	+	+	+	+	+
2	+	+	+	+	+
3	+	+	+	+	+
4	+	+	+	+	-
5	+	+	+	+	-
6	+	-	-	-	-
7	+	-	-	-	-
8	+	-	-	-	-
9	+	-	-	-	-
10	+	-	-	-	-
From healthy foals (n=6)	-	-	-	-	-
NCTC 8239	+	+	+	+	+
a: at least a 75% reduction in toxicity in a duplicate test with antiserum to enterotoxin					

Table 25 Univariate analysis of the numbers of <i>C. perfringens</i> cfu's per gram of faeces isolated from foals with diarrhoea and healthy foals not-in-contact with cases of diarrhoea.						
Isolation Method / Number of cfu per gram of faeces*	Number of positive faecal samples	Number of positive faecal samples in faeces from not-in-contact foals	Odds Ratio	p - value	95% Confidence limits	Number of foals with PCR positive isolates
Direct plate culture 10 ³	56	7	2.49	0.025	1.05 - 6.22	6
Direct plate culture 10 ⁴	22	0	infinity	0.006	~	4
Direct plate culture 10 ⁵	18	1	6.05	0.048	0.84 - 123.49	3
Direct plate culture 10 ⁶	12	2	1.78	0.456	0.36 - 11.96	3
Direct plate culture >10 ⁶	14	0	infinity	0.027	~	3
Heat treatment 10 ³	46	3	4.82	0.005	1.40 - 19.87	3
Heat treatment 10 ⁴	17	1	5.38	0.070	0.74 - 110.06	2
Heat treatment 10 ⁵	9	2	1.18	0.840	0.22 - 8.32	1
Heat treatment 10 ⁶	6	0	infinity	0.157	~	1
Heat treatment >10 ⁶	8	1	2.35	0.412	0.05 - 18.92	3
Alcohol treatment 10 ³	38	1	3.62	0.195	0.47 - 76.46	3
Alcohol treatment 10 ⁴	14	1	1.27	0.824	0.15 - 28.03	2
Alcohol treatment 10 ⁵	7	0	infinity	0.411	~	4
Alcohol treatment 10 ⁶	4	1	0.29	0.277	0.02 - 7.87	2
Alcohol treatment >10 ⁶	3	0	infinity	0.590	~	0
* : The number of colony forming units were rounded up to the nearest power of ten.						
~ : 95% confidence limits could not be calculated.						

Table 26 Univariate analysis of the association of PCR positive <i>C. perfringens</i> with large numbers of cfu's per gram of faeces ($\geq 10^4$) isolated by three different methods.			
Isolation method of PCR positive <i>C. perfringens</i>	Odds Ratio	p - value	95% Confidence limits
Direct plate culture	2.00	0.158	0.69 - 5.84
Heat treatment	3.00	0.103	0.60 - 14.59
Alcohol treatment	7.05	0.003	1.44 - 36.51

9.0 CHAPTER SIX

DISCUSSION

9.1 CONTENTS

9.2 Discussion.....	115
9.2.1 Isolation of <i>Clostridium perfringens</i> from foal faeces	115
9.2.2 Detection of <i>Clostridium perfringens</i> and other pathogens in association with diarrhoea	117
9.2.3 Enterotoxin and equine <i>Clostridium perfringens</i>	122
9.2.4 <i>Clostridium perfringens</i> as a cause of foal diarrhoea	125

9.2 DISCUSSION

Methods designed to increase the sensitivity of *C. perfringens* isolation were developed to help assess the rôle of these bacteria in foal diarrhoea. A broad differential microbiological investigation revealed a strong association between disease and *C. perfringens*. Investigation of the rôle of these bacteria was extended by detection of enterotoxin production and assessment of its association with diarrhoea. This has led to speculation on the pathogenic rôle of *C. perfringens* and foal diarrhoea and in particular on novel pathogenic mechanisms for these bacteria.

9.2.1 Isolation of *Clostridium perfringens* from foal faeces

Clostridium perfringens has the potential to exist in animal faeces in many states including the vegetative cell, the sporulating cell, the endospore of various stages of maturity and the germinating endospore. This variation could be compounded by differences in conditions that affect the ability of endospores to survive and germinate from different strains (Koransky, *et al.*, 1978; Smith & Williams, 1984; Ando *et al.*, 1985). Any single method of culture particularly aimed at the recovery of endospores can be expected to isolate the aetiological agent from most of the affected individuals in outbreaks of human food poisoning; large numbers of *C. perfringens* are consumed which later sporulate. However, for a hypothetical infectious diarrhoea of an unknown pathogenic mechanism (perhaps not related to an intestinal sporulation event), which nonetheless involved *C. perfringens*, any individual method might fail to detect an epidemiological association because it could not isolate the appropriate form, or forms, of these bacteria. Published methods for the recovery of *C. perfringens* endospores are not consistent and just one method has been most often employed for each survey of diarrhoea, and often a different method in each (Hobbs *et al.*, 1953; Sutton & Hobbs, 1968; Koransky, *et al.*, 1978; Borriello, *et al.*, 1985; Brett *et al.*, 1992).

The germination and growth of endospores of different ages were shown here to be marginally favoured by different pretreatment methods. Heating at lower temperatures for shorter times seemed to favour younger endospores. Alcohol

treatment alone or combined with heat offered no advantage over heat treatment alone but when these treatments were combined together with an EDTA recovery period the greatest proportion of endospores were stimulated to germinate and grow. It should be noted that the effect of an EDTA recovery period is completed when used in combination with lysozyme in the solid medium (unpublished observation; in agreement with the observations of Adams, 1973b); combination of these agents are thought to change the structure of the endospore coat and stimulate germination. None of the pretreatments effectively enhanced recovery of the strain which produces heat sensitive endospores. These results indicated that sensitive detection of *C. perfringens* in animal faeces would require the use of several methods designed to recover these bacteria in their different physiological states.

Five different methods used in combination on each sample isolated *C. perfringens* from a much greater proportion of foal faeces than any single method used alone. This confirmed that these bacteria are present in a variety of forms, the isolation of which is favoured by one method or another. Vegetative cells or heat sensitive endospores would not be recovered by any of the methods employing heat, but longer heat treatments might favour the recovery of *C. perfringens* in samples containing predominantly older endospores. Additionally, a pre-enrichment as opposed to direct culture on solid medium might favour the germination of some endospores but not others.

I concluded that it would be necessary to employ a range of methods to isolate *C. perfringens* in a microbiological assessment of the association of these bacteria with a complex disease such as foal diarrhoea. In this way, significant associations might be revealed without dependence on assumptions about the mechanisms by which *C. perfringens* caused disease in relation to a sporulation event and the presence of large numbers of heat resistant endospores of a given maturity.

9.2.2 Detection of *Clostridium perfringens* and other pathogens in association with diarrhoea

There have been very few large scale surveys of potential pathogens and foal diarrhoea (Tzipori, 1985b; Dwyer *et al.*, 1990; Browning *et al.*, 1991; Holland *et al.*, 1991) but, in common with this survey, all have found an association with rotavirus. Also in common with this survey, *Salmonella sp.* were infrequently encountered but when found were isolated from cases of severe disease or in-contact animals: whilst there were too few isolations to give statistical significance to an association with diarrhoea, an association was detected in this survey between isolation from diarrhoea and a fatal outcome. The serotype of the *Salmonella sp.* detected in this survey, namely *S. typhimurium*, was the same as that isolated from most cases of foal salmonellosis (Smith *et al.*, 1978; Browning *et al.*, 1991).

Multivariate analysis and use of healthy not-in-contact animals as the control group has been used here for the first time in a microbiological survey of foal diarrhoea where more potential pathogens were investigated than in previous studies. The previous studies have apparently ignored the affects of sub-clinical infections and used healthy in-contact foals as their control group, thereby weakening any true statistical association with diarrhoea; animals in-contact with diarrhoea would be more likely to ingest and passively excrete pathogens and might weaken any statistical association with diarrhoea.

C. perfringens was revealed for the first time, by the approaches adopted here, to be significantly associated with foal diarrhoea. The only other large survey to investigate the rôle of these bacteria used direct culture and faeces samples were kept for up to a week before testing (Browning *et al.*, 1991), thus reducing the chance of isolating these bacteria.

Examination of the group sample size showed that generally only 2-3 samples were received per location over any three week period. This suggests that either there were no outbreaks and only sporadic cases or that there were outbreaks from which only sporadic cases were submitted. The latter may have been the case for foals at

stud when it is possible that the trouble to submit further samples might not have been taken by veterinarians and stud farm managers once a pathogen was reported from the first sample. This may have been particularly so when rotavirus was detected which is generally accepted as a cause of outbreaks of foal diarrhoea. Nonetheless, the association of *C. perfringens* with diarrhoea was so strong that it is unlikely that it would be reduced by further submissions of rotavirus positive samples which would only serve to increase the overall and relative prevalence of rotavirus.

It is possible to estimate the proportion of diarrhoea caused by each pathogen utilising a calculation of the 'population attributable fraction', or PAF, (Rothman, 1986) which is based on the relative risk and overall prevalence statistics. However, in view of the broad 95% confidence limits on the Odds Ratio (Table 11), which is closely related to the relative risk, and the possible bias introduced by a failure to submit further samples from an outbreak after a rotavirus detection, the PAF was not determined because of its potential to mislead.

The accuracy of the method chosen for detecting a pathogen can greatly influence the results obtained. There are usually a number of methods of detection for widely recognised pathogens; for example, rotavirus can be detected by culture, electrophoresis, electron microscopy, and latex agglutination. However, a balance of economy, speed, and accuracy had to be made in this study for the previously recognised pathogens and so the simple, cheaper, quicker latex agglutination method was chosen for identifying the presence of rotavirus. All isolates of equine rotavirus to date have possessed the group A typing antigen (Snodgrass & Browning, 1991) and consequently, the commercial tests based on the detection of this antigen would be likely to detect the vast majority of these infections.

This survey intentionally employed a variety of techniques for the isolation of *C. perfringens*. These bacteria may exist as vegetative cells or as endospores with different properties regarding conditions for their activation or germination and hence recovery. Each technique would likely to favour the recovery of *C. perfringens* in different states. The possibility exists that there is a difference between strains of

C. perfringens which cause diarrhoea and those which can be isolated from healthy animals. The analysis of the association with diarrhoea and the different isolation methods showed that isolation of *C. perfringens* by heat enrichment was more associated with diarrhoea than any other method. Furthermore, isolation by alcohol treatment was negatively associated with diarrhoea, when using the healthy not-in-contact foals as the control group. This disparate distribution of *C. perfringens* with different isolation properties would support the contention that there are different strains of *C. perfringens* with pathogenic properties, perhaps linked to isolation properties, associated with diarrhoea and other strains which tend to have a more commensal rôle. A test better able to differentiate between these might reveal a stronger association between diarrhoea and the pathogenic strains than with *C. perfringens per se*.

Although *C. perfringens* was widely prevalent amongst and associated with foals with diarrhoea, large numbers of endospores or vegetative forms of these bacteria (greater than 10^3 cfu per ml of faeces) were not frequently encountered as would be expected for most diarrhoea caused by these bacteria in man which is largely food-borne. *C. perfringens* has been described as an infectious cause of diarrhoea in man (Borriello *et al.*, 1984; Borriello, 1988), but in such cases, smaller numbers of these bacteria may be encountered than in cases of food poisoning. This depends on the normal *C. perfringens* carriage rate of the population studied (Brett *et al.*, 1992). If diarrhoea in foals is caused by enterotoxigenic *C. perfringens*, it may be more like the human infectious condition.

The epidemiological analysis when repeated by comparing scouring with all healthy foals, including healthy in-contact animals, still revealed a significant association between *C. perfringens*, rotavirus, *Cryptosporidium sp.* or *S. westeri* and diarrhoea even though the carriage rate amongst the healthy in-contact foals was greater than that in not-in-contact foals. This particularly applied to rotavirus and *C. perfringens*. The greater carriage rate in healthy in-contact foals implied that healthy foals picked up pathogens from their contacts with diarrhoea or the

environment which had become heavily contaminated and then excreted them. Where this is true, surveys of insufficient size that do not distinguish between healthy in-contact and not-in-contact foals may fail to detect an association with diarrhoea. Nonetheless, in this survey the significance of calculations using in-contact animals implied that the association with diarrhoea was real and not an artefact of sampling populations potentially differing in features other than diarrhoea such as the natural incidence of commensal carriage of *C. perfringens*.

C. perfringens in addition to *Salmonella sp.* was also associated with diarrhoea with a fatal outcome. Unfortunately, details of gross pathology were not available but a question arises as to whether there was evidence of severe necrosis of any part of the intestinal mucosa expected of *C. perfringens* enterotoxaemias described in many other mammals, including the horse. The isolates from fatal cases may differ from those from cases of milder diarrhoea by belonging to types B, C or D which are more generally associated with severe enteric disease.

E. coli was isolated from more than 94% of all foals regardless of health status. It was isolated from fewer foals in one study (Holland *et al.*, 1991) and those isolates with any of the virulence factors previously identified for other mammals, were more commonly found in healthy foals in another study (Browning *et al.*, 1991). Healthy in-contact foals were not differentiated from healthy not-in-contact foals, in this latter study, which may explain the failure to associate pathogenic *E. coli* with diarrhoea. In the survey described here, potentially pathogenic *E. coli* were not differentiated and the possibility yet remains that there are strains of these bacteria which have virulence determinants, perhaps specific to the horse, which remain to be described and which, if detected, would be associated with diarrhoea.

Cryptosporidium sp. has for the first time been confirmed as an enteropathogen statistically associated with diarrhoea in foals. Failure of previous studies to detect this association may be due to a lack of differentiation between healthy in-contact and not-in-contact foals. Other studies have shown *Cryptosporidium sp.* to be a cause of diarrhoea in other mammals as well as a possible

cause of diarrhoea in foals, in particular, in immunodeficient foals (Black, 1985; Tzipori, 1985c; Snodgrass *et al.*, 1986; Coleman *et al.*, 1989; Austin *et al.*, 1990; Xiao & Herd, 1994); these studies did not differentiate between healthy in-contact and not-in-contact subjects, so the true association with diarrhoea in these populations may have been greater.

Samples from non-thoroughbred foals not-at-stud which were healthy and not-in-contact with cases were too few to analyse. This population is characterised by very few foals cared for on small private premises and as a consequence, the veterinarian only becomes aware of their existence when they become ill. Collection of samples from healthy foals, therefore, would be expected to be infrequent. Similar difficulties were encountered with foals of all types in parts of the country outside of East Anglia where our laboratory is based. Consequently, it was necessary to amalgamate the areas of the country outside of East Anglia together and when this was done the main findings of the survey held up separately for both for East Anglia and for the rest of the country.

The reduction in odds ratio for the association of *C. perfringens* with diarrhoea outside of East Anglia (although still significant) was probably caused by the need to use all healthy controls since there were insufficient not-in-contact controls to calculate a result. Overall, the prevalence of *C. perfringens* in healthy in-contact foals was significantly greater than that in not-in-contact foals and would have led to an underestimate of its strength of association with disease.

It could be argued that multivariate analysis is not an appropriate method for the kind of data generated in this study largely because it cannot be claimed that all the causes of foal diarrhoea are known and detected and, consequently, an important influence on the logistic regression model may be missing. Indeed, all of the agents associated with diarrhoea by univariate analysis were associated by multivariate analysis with similar strength. The multivariate analysis, however, did take account of the influence of the detected agents on the association with diarrhoea by each and, for example, ruled out the possibility that *C. perfringens* was associated with diarrhoea

because it grew favourably in the gut affected by rotavirus and so may have been associated with diarrhoea in a non-pathogenic way.

It is interesting that most of the diarrhoea associated with *C. perfringens* was mild and self limiting, although as already noted these bacteria were also significantly associated with a large proportion of the fatal cases seen. Presumably, if *C. perfringens* was the cause of the mild disease the mechanism of pathogenicity would be unlike that in the necrotic syndromes caused by types B, C and D. The possibility existed that the enterotoxin was involved which causes a relatively mild diarrhoea in man.

9.2.3 Enterotoxin and equine *Clostridium perfringens*

The epidemiological analysis indicated that *Clostridium perfringens* was significantly associated with diarrhoea in foals which was generally not life threatening. The enterotoxin produced by *C. perfringens* which causes a relatively mild diarrhoea in humans (Stringer, 1985) prompted our search for this determinant in isolates from foal diarrhoea and enterotoxin detected in sporulated culture supernatant fractions of *C. perfringens* by the RPLA test was significantly associated with diarrhoea in foals (see Chapter Four).

The sequence for the enterotoxin gene cloned from one equine isolate which produced particularly large amounts of toxin detected by RPLA and vero cell assay proved to be essentially identical to that previously published for a human isolate, with the exception of the intentional substitution in amino acid residue 2 and an inconsistency at amino-acid residue 298 where 2 of the 3 clones have the base thymidine and the third, cytosine. This is the first confirmation of this sequence and it is likely that the differences with the previously published sequence (Czeczulin *et al.*, 1993) may be artifactual (one created intentionally the other by PCR error).

C. perfringens enterotoxin is produced in large amounts during sporulation of enterotoxigenic wild-type strains of *C. perfringens*, however, expression of the *C. perfringens* enterotoxin by the *E. coli* pTrc 99A recombinant was not sporulation dependant. The recombinant enterotoxin was produced in larger amounts than by the

sporulating *C. perfringens* isolate from which the gene was cloned. The expression was further enhanced by induction of the lac Z promotor. The recombinant would make an excellent starting place for toxin purification with freedom from sporulation dependence and other contaminating clostridial toxins. Nonetheless, expression of a protein by the recombinant which reacted convincingly in the RPLA test and which was cytotoxic for verocells and neutralisable by antiserum produced to enterotoxin, helped to confirm that the fragment cloned from the equine *C. perfringens* was indeed the enterotoxin gene. See Addendum 3 (p153)

The proportion of *C. perfringens* in which enterotoxin was detected by RPLA represented approximately 25% of foals with diarrhoea. However, they represented only 45% of foals with diarrhoea from which *C. perfringens* was isolated. Sporulation, to which enterotoxin production is linked, is an unreliable phenomenon during *in vitro* culture. Consequently, it was possible that part of the large discrepancy between isolation of *C. perfringens* and enterotoxin detection was related to the efficiency of sporulation. The cloning and sequencing of the enterotoxin gene from an equine isolate provided the opportunity to assess the possession of the enterotoxin gene independent of sporulation. It was presumed that possession of the gene would be more common than detection of the enterotoxin.

Surprisingly, considerably fewer isolates reacted in the PCR test for the enterotoxin gene than reacted in the RPLA immunoassay. Comparison of the RPLA with the PCR test and other genetically based tests showed that the RPLA was frequently positive without evidence for the presence of the enterotoxin gene. The results of detection of the enterotoxin gene taken together suggested three principal conclusions.

Firstly, the RPLA test is likely to overestimate the number of isolates that are enterotoxigenic. The false positive reactions in the RPLA may relate to the unintentional detection of other *C. perfringens* toxins or antigens. The RPLA test applied to faeces during the survey of foal diarrhoea was also significantly associated with diarrhoea. However, given the results described here, this may not be surprising

since *C. perfringens* was also associated with diarrhoea and was isolated from 57% of diseased animals. Faeces may have contained the antigens responsible for false positive reactions.

Secondly, *C. perfringens* with the enterotoxin gene represent a minor proportion (5%) of these bacteria associated with foal diarrhoea. Nonetheless, they were probably a cause of diarrhoea since they were statistically associated with disease. Since they were not among those isolated from diarrhoea with a fatal outcome they are, as expected, likely to be a cause of relatively mild disease (compared with the clostridial enterotoxaemias). It remains to be determined whether the disease is contracted directly from other infected foals or if it is acquired from the environment. Given that the environment of the horse is contaminated with faeces along with the coprophagic tendency of foals, the distinction between infectious enterotoxigenic diarrhoea and 'food-borne' diarrhoea made with human disease may not be so clear. Even if acquired from the environment the lower frequency of detection of enterotoxigenic *C. perfringens* suggests a close association with animals carrying these bacteria and diarrhoea. It is noteworthy that diarrhoea caused by these bacteria could be diagnosed by a genetically based test but not reliably by the RPLA test which nonetheless might serve as a relatively simple rapid screen which requires confirmation. The results of a previously reported PCR method, for the detection of the *C. perfringens* enterotoxin gene, agreed with the results of the RPLA test (Saito *et al.*, 1992). However, the agreement of the PCR and RPLA results for the samples selected in their study may reflect the nature of the mechanism of pathogenesis. The recognised mechanism of pathogenesis of *C. perfringens* food-poisoning, with a single sporulation event, would at the same time produce large amounts of enterotoxin which is more likely to give a positive reaction with the RPLA method as well as predispose to isolation of genuinely enterotoxigenic strains than might be the case for other possible mechanisms of pathogenesis which may produce enterotoxin sporadically.

Finally, the strength of association of *C. perfringens* with foal diarrhoea, the high proportion of cases of diarrhoea from which these bacteria were isolated (57%) and the low proportion with the enterotoxin gene (5%) implies that most of these bacteria are a cause of diarrhoea with a mechanism of pathogenesis independent of the enterotoxin. Less than 5% of the cases of diarrhoea studied had a fatal outcome and although *C. perfringens* was also associated with this condition, the majority of diarrhoea associated with these bacteria was clearly not life threatening. A common and striking feature of enterotoxaemic clostridial diarrhoea described in other species, as well as numerous case reports of affected horses, is extensive necrosis of the intestinal mucosa which is almost inevitably fatal. Consequently, most of the foal diarrhoea associated with *C. perfringens* must involve a novel mechanism and, perhaps, novel undescribed enterotoxins. Currently, no test is available to differentiate the foal enteropathogenic *C. perfringens* from *C. perfringens* isolated from healthy foals. Study of the genes encoding the wide range of toxins seen in different strains of *C. perfringens* from different backgrounds may reveal a pattern found in isolates from foal diarrhoea rarely seen in strains from healthy animals.

9.2.4 *Clostridium perfringens* as a cause of foal diarrhoea

Conventional techniques for *C. perfringens* isolation (heat activation at 70°C to 80°C, for 20 minutes; Tsai & Riemann, 1974, or alcohol treatment with 50% ethanol for 1 hour; Koransky *et al.*, 1978) have not previously revealed an association of *C. perfringens* with foal diarrhoea (Browning *et al.*, 1991). However, additional methods used in this study reveal an association of *C. perfringens* with diarrhoea in foals (Chapter Three). This suggests that there is probably a mechanism of pathogenesis for *C. perfringens* in foals, different from that recognised in traditional human food-poisoning, where large numbers ($>10^6$ cfu / g of faeces) of endospores and the presence of enterotoxin are considered as diagnostic (Hobbs *et al.*, 1953; Sutton and Hobbs, 1968; Koransky, *et al.*, 1978; Borriello *et al.*, 1985; Brett *et al.*, 1992). Therefore, identification of *C. perfringens* as the cause of foal diarrhoea is not solely dependant on isolating large numbers of *C. perfringens*

endospores, as *C. perfringens* present in the vegetative state or low numbers of endospores may also be indicative of a *C. perfringens* infection.

Recently, other mechanisms of pathogenesis for enterotoxigenic *C. perfringens* producing diarrhoea and / or death have been recognised in humans, for example, antibiotic-associated diarrhoea (Borriello *et al.*, 1984; Borriello *et al.*, 1985; Borriello & Larson, 1985; Williams *et al.*, 1985; Samuel *et al.*, 1991); sporadic infectious diarrhoea (Borriello, 1985; Borriello *et al.*, 1987; Borriello, 1988; Larson & Borriello, 1988; Brett *et al.*, 1992); and sudden infant death syndrome (SIDS) (Murrell *et al.*, 1987; Murrell *et al.*, 1993). Some of the cases observed have large numbers of *C. perfringens* present in the faeces but these observations are mostly made in the elderly institutionalised patient. Often, healthy members of this population carry large numbers of *C. perfringens* as commensals (Yamigoshi *et al.*, 1976) and it has been suggested that large numbers of *C. perfringens* are not so important in the diagnoses of this disease (Brett *et al.*, 1992), as they are in human food poisoning. The presence of *C. perfringens* enterotoxin in the faeces remains a more important factor (Borriello, 1988; Brett *et al.*, 1992).

It was of interest that there was no obvious association between the presence of large numbers of *C. perfringens* ($>10^6$ cfu / g of faeces) and foal diarrhoea. This is in stark contrast to the situation that prevails in human enterotoxigenic *C. perfringens* food poisoning. Although there is a question mark over the diagnostic value of detecting large numbers of *C. perfringens* after alcohol or heat treatment from cases of infectious diarrhoea in man caused by enterotoxigenic strains, an overall association with large numbers has nonetheless been noted. These results imply that if enterotoxigenic strains are largely responsible for the association of *C. perfringens* with foal diarrhoea, then (a) the elaboration of enterotoxin may be not so dependent on sporulation as it is in human disease; (b) the horse gut may be considerably more sensitive to the action of the enterotoxin than the human gut; or (c) the gut of the horse may not permit the survival of large numbers of endospores quite so readily as the human gut.

Discovery that the majority of *C. perfringens* isolated from cases of foal diarrhoea did not contain the enterotoxin gene (by PCR method) was surprising given the strength of association of these bacteria with foal diarrhoea and their prevalence. Nonetheless, those *C. perfringens* with the enterotoxin gene were almost exclusively isolated from cases of diarrhoea. Furthermore, there was a strong association between the isolation of enterotoxigenic *C. perfringens* and recovery of large numbers of *C. perfringens* following alcohol treatment. This supported the case that enterotoxigenic *C. perfringens* are a cause of foal diarrhoea and that sporulation may play a part in pathogenesis just as it does in human disease. However, as with infectious enterotoxigenic *C. perfringens* diarrhoea in man, large numbers of these bacteria may not be diagnostically significant (Brett *et al.*, 1992). An interesting finding was that two of the four faeces samples with blood came from scouring foals with enterotoxigenic *C. perfringens*; blood in faeces has been noted in association with human infectious and food borne enterotoxigenic *C. perfringens* diarrhoea (Larson & Borriello, 1988).

Enterotoxigenic *C. perfringens* from only 3 of eleven foals positive for these bacteria were serotypable. This is in contrast with strains typed from a series of 1444 proven outbreaks of human food poisoning where at least 85% were typable (Moir Brett, Food Hygiene Laboratory, Colindale, personal communication of research in preparation for publication). The serotyping system was developed for human food poisoning isolates (Hobbs *et al.*, 1953; Stringer *et al.*, 1980) although most isolates from cases of infectious enterotoxigenic *C. perfringens* were also typable (Brett *et al.*, 1992). The large proportion of untypable equine enterotoxigenic *C. perfringens* suggest that they are epidemiologically unlinked to human food poisoning and perhaps that they may be specifically associated with horses. The serotypable isolates from the three other foals were, however, of serotypes that are neither particularly common nor rare in association with human food poisoning.

Why enterotoxigenic *C. perfringens* should be associated with the isolation of large numbers of these bacteria after alcohol treatment when isolation of all

C. perfringens by this method is negatively associated with diarrhoea compared with in-contact foals is unclear. Perhaps this method of isolation favoured the growth of at least two populations of *C. perfringens*, the largest of which was most commonly found in healthy foals and the much smaller mostly found in foals with diarrhoea.

Enterotoxigenic *C. perfringens* have been detected in horses on three previous occasions. On the first it was detected in one case of foal diarrhoea although the methods of detection were poorly described (Reed et al., 1983). On the second occasion it was reported both in healthy and scouring adult horses using inoculation of faecal extracts into mouse ligated loops and neutralisation by antiserum (Ehrich et al., 1984); it is difficult to be certain of the specificity of the antiserum used. On the last occasion the gene was detected by gene probe in over half of the isolates from horses but no comment was made of their disease status (Tschivdewhan et al., 1991). All of these findings are difficult to comment on in relation to the research described in this thesis in view of their approach and scant detail.

There was no association between enterotoxigenicity and large numbers of *C. perfringens* after heat treatment. Nonetheless, there was a more significant association between diarrhoea and the isolation of any number, small or large, of *C. perfringens* after heat treatment, than by any other isolation method. This discrepancy is difficult to understand but may be related to the strength of association between foal diarrhoea and *C. perfringens* which is accounted for largely by non-enterotoxigenic bacteria. These strains of non-enterotoxigenic *C. perfringens* may have a mechanism of pathogenicity which is independent of toxins formed during sporulation and hence an association between isolation and disease would not be dependent on endospore numbers.

There are three possible explanations to consider with regard to the association of non-enterotoxigenic *C. perfringens* with foal diarrhoea. The first is that *C. perfringens* is not involved in the pathogenicity of diarrhoea but that it finds the environment of the abnormal gut favourable to thrive in. If this were so a statistical interaction would have been expected between *C. perfringens* and other causes of

diarrhoea such as rotavirus. This kind of interaction was not present and so this possibility seems unlikely. However, antibiotic treatment is known to favour the multiplication of *C. perfringens* in man (Borriello *et al.*, 1984; Borriello *et al.*, 1985; Borriello & Larson, 1985; Williams *et al.*, 1985; Samuel *et al.*, 1991) and possibly in the horse (Andersson *et al.*, 1971). Since foal diarrhoea is often treated with these drugs, even though they are not indicated, this may provide an explanation for a non-pathogenic association with diarrhoea. If this were true isolation of *C. perfringens* from healthy and scouring foals would be equally likely by different methods. However, isolation after alcohol treatment was more likely from healthy foals than after heat treatment which was more likely from scouring foals. This suggested that *C. perfringens* with different properties were isolated from the different populations of foal which probably could not be explained by treatment by a variety of different drugs and dosing regimes; in other words, there is unlikely to be a single selective pressure encompassed by the antibiotic treatment given to foals.

A second possibility is that the same kinds of *C. perfringens* can be found in scouring and healthy foals and that disease is caused by an undetermined predisposing factor favouring the proliferation of these bacteria which then elaborate a variety of toxins which together cause diarrhoea. Again, however, this possibility does not accommodate the finding that isolation after heat treatment was more likely from scouring foals and than isolation after alcohol treatment which was associated with healthy foals.

The third possible explanation is that there are strains of *C. perfringens* which are specially adapted to cause diarrhoea in the foal. This might account for the uneven distribution of the isolation of *C. perfringens* by different methods if one of the methods in some way favoured the isolation of strains with the pathogenic property. Presumably, adaptation of strains to cause diarrhoea would have a molecular basis in terms of pathogenic determinants such as a toxin or a specially adapted form of a toxin produced by most *C. perfringens* involved. Enterotoxigenic *C. perfringens* have already been discussed above but these accounted for a minority

of *C. perfringens* isolated from scouring foals. A protein antigenically related to enterotoxin has been described which is present in some non-enterotoxigenic strains (Ryu & Labbé, 1993). Perhaps this molecule, or one like it, could act as an enterotoxin. The PCR method of amplification of the enterotoxin gene may have missed the gene for this protein because of mismatch of one or both of the primers. If this protein is widely spread amongst non-enterotoxigenic strains from foals it would explain the discrepancy between the RPLA antigenically based test and the PCR.

Other possible differences in molecular adaptation might explain a pathogenic association with foal diarrhoea. *C. perfringens* of types A, B, C and D have been isolated from case reports in horses (Montgomerie and Rowlands, 1937; Mason & Robinson, 1938; Dickie *et al.*, 1978; Wierup & DiPietro, 1981; Niilo & Chalmers, 1982; Sims *et al.*, 1985; Howard-Martin *et al.*, 1986; Pearson *et al.*, 1986; Niilo, 1987; Dart *et al.*, 1988; Stubbings, 1990). Almost all of these have been associated with aggressive lesions of the gut with extensive necrosis and blood in the faeces. Most of the faeces from scouring foals did not contain blood, although it is not possible to say that these cases did not go on to develop these signs. Two of the four faeces with blood were from post mortem samples which contained *C. perfringens*. Most case reports of equine disease associated with types B and C were in animals less than one week old. It is interesting that a large proportion of *C. perfringens* from fatal cases were in foals of this age, although there were too few samples to establish a statistical link between isolation and age group. If these isolates were to be typed, however, a stronger association between age, fatal diarrhoea and specific types of *C. perfringens* might be revealed. The possibility that the majority of foal diarrhoea is a mild form of the more aggressive *C. perfringens* disease described in case reports cannot be ruled out for the moment. There is an urgent need to classically type the equine isolates from this survey using the published gene sequences for the major toxins (Titball *et al.*, 1989; Hunter *et al.*, 1992; Hunter *et al.*, 1993; Perelle *et al.*, 1993) by a genetically based method such as PCR which has recently been used successfully (Daube *et al.*, 1994).

If the distribution of the enterotoxin related antigen or classical toxins does not explain the association between foal diarrhoea and *C. perfringens* then the answer must be sought elsewhere. Genome mapping of *C. perfringens* has shown that the first 20% of DNA downstream of the putative origin of replication not only contains all of the virulence determinants that were mapped but also the greatest inter-strain variation (Canard *et al.*, 1992). The authors of this work have suggested that this clustering of variation together with virulence determinants may hold the key to understanding why particular strains cause individual syndromes in different species of animal. It may be worth investigating restriction fragment length polymorphisms with labelled virulence genes to see if a particular pattern is more strongly associated with foal diarrhoea than *C. perfringens* generally.

In conclusion, a clear association between *C. perfringens* and a large proportion of foal diarrhoea has been discovered. Enterotoxigenic *C. perfringens* were also strongly associated with diarrhoea but they represented too small a proportion of *C. perfringens* from cases to account for the overall association. There is now a need to investigate molecular differences between isolates from diarrhoea and those from healthy foals. This may help to explain the association of *C. perfringens* with diarrhoea as well as provide the basis for methods of diagnosis and to identify strains for more detailed pathogenic studies.

10.0 REFERENCES

- Ababouch, L., Dikra, A. & Busta, F.F.** (1987). Tailing of survivor curves of clostridial spores heated in edible oils. *J. Appl. Bact.* **62**, 503-511.
- Adams, D. M.** (1973a). Inactivation of *Clostridium perfringens* type A at ultra high temperatures. *Appl. Micro.* **26**, 282-287.
- Adams, D. M.** (1973b). Sensitisation by ethylene diamine tetra acetate of *Clostridium perfringens* type A spores to germination by lysozyme. *J. Bact.* **116**, 500-502.
- Adams, D. M.** (1974). Requirement for and sensitivity to lysozyme by *Clostridium perfringens* spores heated at ultra high temperatures. *Appl. Micro.* **27**, 797-801.
- Adams, D. M.** (1978). Heat injury of bacterial spores. *Adv. Appl. Micro.* **23**, 245-261.
- Agfa-Gevaert Ltd.**, 27, Great West Road, Brentford, Middlesex, U.K..
- Amersham International**, Amersham Place, Little Chalfont, Bucks., HP7 9BR.
- Andersson, G., Ekman, L., Mansonn, Z., Personn, S., Rubarth, S. & Tufvesson, G.** (1971). Lethal complications following administration of oxytetracycline in the horse. *Nord. Vet-Med.* **23**, 9.
- Ando, Y.** (1978). Biochemical mechanisms of ionic germination of spores of *Clostridium perfringens* type A. In *Spores VII*, pp. 75-79. Edited by G. Chambliss & J. C. Vary. Washington, D.C.: American Society for Microbiology.
- Ando, Y.** (1979). Spore lytic enzyme released from *Clostridium perfringens* spores during germination. *J. Bact.* **140**, 59-64.
- Ando, Y. & Tsuzuki, T.** (1984). Energy-dependant activation of spore-lytic enzyme precursor by germinated spores of *Clostridium perfringens*. *Biochem. Biophys. Res. Commun.* **123**, 463-467.
- Ando, Y., Tsuzuki, T., Sunagawa, H. & Oka, S.** (1985). Heat resistance, spore germination and enterotoxigenicity of *Clostridium perfringens*. *Micro. Immun.* **29**, 317-326.
- Applied Biosystems Inc.**, A division of Perkin-Elmer, Weiterstadt, Germany.

- Austin, S. M., DiPietro, J. A. & Foreman, J. H. (1990).** *Cryptosporidium* sp. : A cause of diarrhoea in immunocompetent foals. *Eq. Pract.* **12**, 10.
- Baker, J. C. & Ames, T. R. (1987).** Total parenteral nutritonal therapy of a foal with diarrhoea from which parvovirus-like particles were identified. *Eq. Vet. J.* **19**, 342-344.
- Barnes, D. M. & Moon, H. W. (1964).** Enterotoxemia in pigs due to *Clostridium perfringens* type C. *J. Am. Vet. Med. Assoc.* **144**, 1391-1394.
- Bergey, D. H. (1994).** Bergey's manual of determinative bacteriology. Edited by J. G. Holt, N. R. Kreig, P. H. Sneath, J. T. Staley & S. T. Williams. Baltimore, Maryland, U.S.A. Williams and Wilkins.
- Biogenesis Limited**, 12 Yeomans Park, Bournemouth, England, BH8 0BJ.
- bioMérieux sa**, 69280 Marcy-l'Etoile, France.
- Bio-Rad Laboratories**, 2000 Alfred Nobel Drive, Hercules, CA 94547.
- Black, R. E. (1985).** Relative importance of enteropathogens affecting humans. In: *Infectious diarrhoea in the young*, pp. 365-370. Edited by S. Tzipori *et al.*. Elsevier Science Publishers.
- Border, M., Firehammer, B. D., Shoop, D. & Myers, L. (1985).** Isolation of *Bacteroides fragilis* from the faeces of diarrheic calves and lambs. *J. Clin. Micro.* **21**, 472-473.
- Borriello, S. P. (1985).** Newly described clostridial diseases of the gastrointestinal tract: *Clostridium perfringens* enterotoxin-associated diarrhoea. In: *Clostridia in gastrointestinal disease*, pp.223-229. Edited by S. P. Borriello. Boca Raton, Florida, USA : CRC Press.
- Borriello, S. P. (1988).** Epidemic *Clostridium perfringens* diarrhoea. *Inf. Focus* **1**, 12.
- Borriello, S. P. (1992).** Colonisation resistance and bacteriotherapy: The *Clostridium difficile* experience. *Med. Lab. Sci.* **49**, 198.
- Borriello, S. P., Barclay, F. E., Welch, A. R., Stringer, M. F., Watson, G. N., Williams, R. K. T., Seal, D. V. & Sullens, K. (1985).** Epidemiology of

- diarrhoea caused by enterotoxigenic *Clostridium perfringens*. *J. Med. Micro.* **20**, 363-372.
- Borriello, S. P. & Carman, R. J.** (1985). Clostridial diseases of the gastrointestinal tract in animals. In *Clostridia in gastrointestinal disease*, pp.195-221. Edited by S. P. Borriello. Boca Raton, Florida, USA : CRC Press.
- Borriello, S. P. & Larson, H. E.** (1985). Pseudomembranous and antibiotic-associated colitis. In: *Clostridia in gastrointestinal disease*. pp.145-164. Edited by S. P. Borriello. Boca Raton, Florida: CRC Press.
- Borriello, S. P., Larson, H. E., Barclay, F. E. & Welch, A. R.** (1987). *Clostridium perfringens* enterotoxin - associated diarrhoea. In: *Recent advances in anaerobic bacteriology* pp.33-42. Edited by S. P. Borriello & J. M. Hardie. Martinus Nijhoff.
- Borriello, S. P., Larson, H. E., Welch, A. R., Barclay, F., Stringer, M. F. & Bartholomew, B. A.** (1984). Enterotoxigenic *Clostridium perfringens* : a possible cause of antibiotic-associated diarrhoea. *Lancet* **1**, 305-307.
- Borriello, S. P., & Williams, R. K. T.** (1985). Treatment of *Clostridium perfringens* enterotoxin-associated diarrhoea with metronidazole. *J. Infect.* **10**, 65-67.
- Bowman, W. C. & Rand, M. J.** (1980). Chemotherapy of infections by multicellular organisms. In: *Textbook of pharmacology*. pp. 37.1-37.13. London: Blackwell Scientific Publications.
- Brett, M. M., Rodhouse, J. C., Donovan, T. J., Tebbutt, G. M. & Hutchinson, D. N.** (1992). Detection of *Clostridium perfringens* and its enterotoxin in cases of sporadic diarrhoea. *J. Clin. Pathol* **45**, 609-611.
- Bridson, E. Y.** (1990). The Oxoid Manual. Basingstoke, Unipath Ltd.
- Browning, G. F., Chalmers, R. M., Snodgrass, D. R., Batt, R. M., Hart, C. A., Ormarod, S. E., Leadon, D., Stoneham, S. J. & Rosedale, P. D.** (1991). The prevalence of enteric pathogens in diarrhoeic thoroughbred foals in Britain and Ireland. *Eq. Vet. J.* **23**, 405-409.

- Browning, G. F., Sykes, J. E., Huntingdon, P. J., Holywell, C. A. & Begg, A. P.** (1992). Rotavirus infections in Australian foals. *Aust. Eq. Vet.*, **10**, 123-126.
- Buecher, V.** (1989). The use of antimicrobial therapy in gastrointestinal disease in the adult horse. *Eq. Pract.* **11**, 10.
- Canard, B., Saint-Joanis, B. & Cole, S. T.** (1992). Genomic diversity and organisation of virulence genes in the pathogenic anaerobe *Clostridium perfringens*. *Molec. Micro.* **6**, 1421-1429.
- Candy, D. C. A., Osborne, M. P., Haddon, S. J., Wallis, T. S., Starkey, W. G. & Stephen, J.** (1985). *Salmonella*. In: *Infectious diarrhoea in the young*. pp.288-293. Edited by S.Tzipori *et al.*. Elsevier Science Publishers BV.
- Carter, M. E., Dewes, H. G. & Griffiths, O. V.** (1979). Salmonellosis in foals. *J. Eq. Med. Surg.* **3**, 78-83.
- Chang, K. Y. & Carr, C. W.** (1971). Studies on the structure and function of lysozyme. I. The effect of pH and cation concentration on lysozyme activity. *Biochem. Biophys. Acta.* **229**, 496-503.
- Clifford & Anellis** (1971). *Clostridium perfringens* I. Sporulation in a biphasic glucose ion-exchange resin medium. *Appl. Microbiol.* **22**, 856.
- Coleman, S. U., Klei, T. R., French, D. D., Chapman, M. R. & Corstvet, R. E.** (1989). Prevalence of *Cryptosporidium* sp. in equids in Louisiana. *Am. J. Vet. Res.* **50**, 575-577.
- Collins, J. E., Bergeland, M. E., Bouley, D., Ducommun, A. L., Francis, D. H. & Yeske, P.** (1989). Diarrhoea associated with *Clostridium perfringens* type A enterotoxin in neonatal pigs. *J. Vet. Diagn. Invest.* **1**, 351-353.
- Conner, M. E. & Darlington, R. W.** (1980). Rotavirus infection in foals. *Am. J. Vet. Res.* **41**, 1699-1703.
- Cowan, S.T.** (1974). Cowan and Steel's Manual for the identification of medical bacteria. Second Edition. Cambridge: Cambridge University Press.

- Craven, S. E.** (1988). Activation of *Clostridium perfringens* spores under conditions that disrupt hydrophobic interactions of biological macromolecules. *Appl. Environ. Micro.* **54**, 2042-2048.
- Craven, S. E. & Blankenship, L. C.** (1985). Activation and injury of *Clostridium perfringens* pores by alcohols. *Appl. Environ. Micro.* **50**, 249-256.
- Craven, S. E. & Blankenship, L. C.** (1987). Changes in the hydrophobic characteristics of *Clostridium perfringens* spores and spore coats by heat. *Can. J. Microbiol.* **33**, 773-776.
- Cruickshank, R., Duguid, J. P., Marmion, B. P. & Dwain, R. H. A.** (1975). Medical Microbiology. The Practice of Medical Microbiology. 12th Edn. **2**, 306-307. Churchill Livingstone.
- Czczulin, J. R., Hanna, P. C. & McClane, B. A.** (1993). Cloning, nucleotide sequencing, and expression of the *Clostridium perfringens* enterotoxin gene in *Escherichia coli*. *Inf. Immun.* **61**, 3429-3439.
- Dart, A. J., Pascoe, R. R., Gibson, J. A. & Harrower, B. J.** (1988). Enterotoxaemia in a foal due to *Clostridium perfringens* type A. *Aust. Vet. J.* **65**, 330-331.
- Daube, G., China, B., Simon, P., Hvala, K. & Mainil, J.** (1994). Typing *Clostridium perfringens* by *in vitro* amplification of toxin genes. *J. Appl. Bact.* **77**, 650-655.
- Dean, A. D., Dean, J. A., Burton, J. H. & Dicker, R. C.** (1990). Epi Info, Version 5 : a word processing, database, and statistics program for epidemiology on micro-computers. Centers for Disease Control, Atlanta, Georgia, USA.
- Dickie, C. W., Klinkerman, D. L. & Petrie, R. J.** (1978). Enterotoxemia in two foals. *Journal of the Am. Vet. Med. Assoc.* **173**, 306.
- Drasar, B. S. & Barrow, P. A.** (1985). Benefit and mischief from the intestinal microflora. In : *Intestinal microbiology*. pp.59-77. London: Van Nostrand Reinhold (UK) Co.Ltd.

- Drasar, B. S. & Duerden, B. I.** (1991). Anaerobes in the normal flora of man. In:
Anaerobes in human disease. Edited by B. I. Duerden & B. S. Drasar. pp.162.
London: Edward Arnold.
- Duncan, C. L. & Strong, D. H.** (1968). Improved medium for sporulation of
Clostridium perfringens. *Applied Microbiology* **16**, 82-89.
- Dwyer, R. M., Powell, D. G., Roberts, A. W., Donahue, J. M., Lyons, E. T.,
Osborne, M. & Woode, G. N.** (1990). Infectious foal diarrhoea : A three year
study of epidemiology and prevention. *Proc. Am. Coll. Vet. Int. Med. Forum* **8**,
569-571.
- Edwards, G. B.** (1992). Diseases of the stomach; in: Equine anaesthesia, abdominal
surgery and medicine of the foal. Proceedings of the fourteenth Bain-Fallen
Memorial Lectures. *Aust. Eq. Vet. Assoc.* **10**, 117-127.
- Ellner, P. D.** (1956). A medium promoting rapid quantitative sporulation in
Clostridium perfringens. *J. Bact.* **71**, 465.
- Evans, D. G.** (1945). The *in vitro* production of alpha-toxin, theta-haemolysin and
hyaluronidase by strains of *Clostridium welchii* type A, and the relationship of *in*
vitro properties to virulence for guinea pigs. *J. Path. Bact.* **57**, 75.
- Firehammer, B. D. & Myers, L. L.** (1981). *Campylobacter fetus* subsp *jejuni* : Its
possible significance in enteric disease of calves and lambs. *Am. J. Vet. Res.* **42**,
918-922.
- Food Hygiene Laboratory, Public Health Laboratory, 61, Colindale Avenue,**
London, NW9 5HT.
- Friebe, W. R. & Duncan, C. L.** (1973). Homology between enterotoxin protein
and spore structural protein in *Clostridium perfringens* type A. *Eur. J. Biochem.*
39, 393-401.
- Genetic Research International, Dunmow, Essex, CM6 3LD.**
- George, W. L. & Finegold, S. M.** (1985). Clostridia in gastrointestinal flora. In:
Clostridia in gastrointestinal disease. Edited by S. P. Borriello. pp.1-37. Boca
Raton, Florida: CRC Press.

GIBCO BRL, Life Technologies, Inc., Gaithersburg, MD 20884-9980 U.S.A.

Gnanasoorian, S. (1992). Detection of *Cryptosporidium* oocysts in faeces: comparison of conventional and immunofluorescence methods. *Med. Lab. Sci.* **49**, 211-212.

Goldner, S. B., Solberg, M., Jones, S. & Post, L. S. (1986). Enterotoxin synthesis by nonsporulating cultures of *Clostridium perfringens*. *Appl. Environ. Micro.* **52**, 407-412.

Granum, P. E., Telle, W., Olsvik, Ø. & Stavn, A. (1984). Enterotoxin formation by *Clostridium perfringens* during sporulation and vegetative growth. *Int. J. Food Microbiol.* **1**, 43-49.

Haldene, J. B. S. (1956). The estimation and significance of the logarithm of a ratio of frequencies. *Annal. Hum. Gene.* **20**, 309-311.

Hanna, P. C., Wnek, A. P. & McClane, B. A. (1989). Molecular cloning of the 3' half of the *Clostridium perfringens* enterotoxin gene and demonstration that this region encodes receptor-binding activity. *J. Bact.* **171**, 6815-6820.

Hardy, M. E., Woode, G. N., Xu, Z., William, J. D., Conner, M. E., Dwyer, R. M. & Powell, D.G. (1991). Analysis of serotypes and electropherotypes of equine rotaviruses isolated in the United States. *J. Clin. Micro.* **29**, 889-893.

Harman, S. M. & Kaulter, D. A. (1987). Enumeration of *Clostridium perfringens* spores in human faeces: comparison of four culture media. *J. Assoc. Off. Anal. Chem.* **70**, 994-996.

Hashimoto, T., Frieben, W. R. & Conti, S. F. (1972). Kinetics of germination of heat-injured *Bacillus cereus* spores. In: *Spores V*. Edited by H. O. Halverson, R. Hanson and L. L. Campbell. pp.409-415. Washington DC: American Society for Microbiology.

Hobbs, B. C., Smith, M. E., Oakley, C. L., Warrack, G. H. & Cruickshank, J. C. (1953). *Clostridium welchii* food poisoning. *J. Hyg.* **51**, 75-101.

- Holland, R. E., Schmidt, A., Sriranganathan, N., Brown, C. M., Walker, R. D. & Wilson, R. A. (1991). Survey of infectious causes of diarrhoea in foals. *Eq. Inf. Dis. (conf)* **6**, 55-60.
- Howard-Martin, M., Morton, R. J., Qualls, C. W. & MacAllister, C. G. (1986). *Clostridium perfringens* type C enterotoxemia in a newborn foal. *J. Am. Vet. Med. Assoc.* **189**, 564-565.
- Hunter, S.E.C, Clarke, I.N., Kelly, D.C. & Titball, R.W. (1992). Cloning and nucleotide sequence of the *Clostridium perfringens* epsilon-toxin gene and its expression in *Escherichia coli*. *Infect. Immun.* **60**, 102-110.
- Hunter, S.E.C, Brown, E., Oyston, P. C. F., Sukarai, J. & Titball, R.W. (1993). Molecular genetic analysis of beta-toxin of *Clostridium perfringens* reveals sequence homology with alpha-toxin, gamma-toxin, and leukocidin of *Staphylococcus aureus*. *Infect. Immun.* **61**, 3959-3965.
- Hybaid Limited, Crown House, 51, Aldwych, London, WC2B 4LS.
- Hyun, H. H., Zeikus, J. G., Langin, R. M., Millet, J. & Ryter, A. (1983). Ultrastructure and extreme heat resistance of spores from thermophilic *Clostridium* species. *J. Bact.* **156**, 1332-1337.
- Invitrogen Corporation, 3985 B Sorrento Valley Blvd., San Diego, CA 92121.
- Iwanejko, L. A., Routledge, M. N. & Stewart, G. S. A. B. (1989). Cloning in *Escherichia coli* of the enterotoxin gene from *Clostridium perfringens* type A. *J. Gen. Micro* **135**, 903-909.
- Jewell, N. P. (1986). On the bias of commonly used measures of association for 2 x 2 tables. *Biometrics* **42**, 351-358.
- Jewkes, J., Larson, H. E., Price, A. B., Sanderson, P. J. & Davies, H. A. (1981). Aetiology of acute diarrhoea in adults. *Gut* **22**, 388-392.
- Johnson, M. W., Fitzgerald, G. R., Welter, M. W. & Weter, C. J. (1992). The six most common pathogens responsible for diarrhoea in newborn pigs. *Vet. Med.* **87**, 382-386.

- Jones, R. L., Adney, W. S., Alexander, A. F., Shideler, R. K. & Traub-Dargatz J.L.** (1988). Hemorrhagic necrotizing enterocolitis associated with *Clostridium difficile* infection in four foals. *J. Am. Vet. Med. Assoc* **193**, 76-79.
- Jones, R. L., Adney, W. S. & Shideler, R. K.** (1987). Isolation of *Clostridium difficile* and detection of cytotoxin in the faeces of diarrhoeic foals in the absence of antimicrobial. *J. Clin. Micro.* **25**, 1225-1227.
- Keynan, A.** (1978). Spore structure and its relations to resistance dormancy and germination. In: *Spores VII*. pp.43-53. Edited by G. Chambliss & J. C. Vary. Washington DC: American Society for Microbiology.
- Kenyan, A. & Evenchick, Z.** (1969). Activation. In: *The bacterial spore*. pp.359-396. Edited by G. W. Gould & A. Hurst. London: Academic Press.
- Kenyan, A., Evenchick, Z., Halvarson, H. O. & Hastings, J. W.** (1964). Activation of bacterial endospores. *J. Bacteriol.* **88**, 313-318.
- Koransky, J. R., Allen, S. D. & Dowell, jr. V. R.** (1978). Use of ethanol for selective isolation of sporeforming microorganisms. *Appl. Environ. Micro.* **35**, 762-765.
- Koterba, A. M.** (1991). Infection control and prevention in the critically ill foal. *Eq. pract.* **13**, 29-32.
- Labbe, R. G. & Duncan, C. L.** (1977). Spore coat protein and enterotoxin synthesis in *Clostridium perfringens*. *J. Bact.* **131**, 713-715.
- LabM**, Topley House, P.O. Box 19, Bury, BL9 6AU.
- Laemmli, U. K.** (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Larson, H. E. & Borriello, S. P.** (1988). Infectious diarrhoea due to *Clostridium perfringens*. *J. Inf. Dis.* **157**, 390-391.
- Levine, M. M.** (1985). Dehydration, metabolic and nutritional consequences of infant diarrhoea and oral rehydration. In: *Infectious diarrhoea in the young*. pp.383-389. Edited by S. Tzipori *et al.* London: Elsevier Science Publishers.

- Levinson, H. S. & Feeherry, F. E. (1978). Kinetics of heat activation of spores of *Clostridium perfringens*. In: *Spores VII*. pp.34-40. Edited by G. Chambliss & J. C. Vary. Washington DC: American Society for Microbiology.
- Long, S. S. & Swenson, R. M. (1977). Development of anaerobic faecal flora in healthy newborn infants. *J. pediat.* **91**, 298-301.
- Lyons, E. T. , Drudge, J. H., Tolliver, S. C. & Granstrom, D. E. (1991a). The role of intestinal nematodes in foal diarrhoea. *Vet. Med.* **86**, 320-328.
- Lyons, E. T., Granstrom, D. E., Drudge, J. H. & Tolliver, S. C. (1991b). The role of intestinal protozoa in foal diarrhoea. *Vet. Med.* **86**, 193-197.
- Madigan, J. E., Walker, R. L., Hird, D. W., Case, J. T., Bogenrief, D. S. & Smith, B. P. (1990). Equine neonatal salmonellosis: clinical observations and control measures (a case report). *Proc. Am. Ass. Equine Pract.* **36**, 371-375.
- M.A.F.F. (Ministry of Agriculture, Fisheries and Food) (1985). A modified McMaster method. In: *Manual of Veterinary Investigation Laboratory Techniques*. **2**, 162-163, HMSO.
- Mahony, D. E., Ahmed, R. & Jackson, S. G. (1992). Multiple typing techniques applied to a *Clostridium perfringens* food poisoning outbreak. *J. Appl. Bact.* **72**, 309-314.
- Martens, R. J. (1979). Non-infectious diarrhoea in the foal. *Proc. Am. Ass. Eq. Pract.* **25**, 205-216.
- Martens, R. J., & Scrutchfield, W. L. (1982). Foal diarrhoea: Pathogenesis, etiology and therapy. *Compend. Contin. Ed. Prac. Vet.* **4**, 200-210.
- Mason, J. H. & Robinson, E. M. (1938). The isolation of *Clostridium welchii* type B from foals affected with dysentery. *Ond. J. Vet. Sci. & Ani. Ind.* **11**, 333-337.
- McDonel, J. L. (1986). Toxins of *Clostridium perfringens* types A, B, C, D and E. In: *International Encyclopaedia of Pharmacology and Therapeutics*. pp.477-517. Edited by F. Dorner & J. Drew. London: Pergamon Press.

- McNulty, M. S.** (1985). Enteric viral infections in young animals. In: *Infectious diarrhoea in the young*. pp.231-239. Edited by S. Tzipori *et al.* London: Elsevier Science Publishers.
- Meduski, J. W. & Hochstein, P. E.** (1972). Hot-cold haemolysis: the role of positively charged membrane lipids. *Experientia*. **28**, 565-566.
- Melvin, D. M. & Healy, G. R.** (1988). Intestinal and urogenital protozoa. In: *Manual of Clinical Microbiology*. 4th Edition. pp. 647. Edited by E. H. Lennette, A. Balows, W. J. Hauder jr. & H. J. Shadomy. Washington D.C., USA: American Society for Microbiology.
- Millipore (U.K.) Ltd.**, The Boulevard, Blackmoor Lane, Watford, Herts., WD1 8YW.
- Mitsubishi electrical (UK) Limited**, made by Mitsubishi electrical corporation, Tokyo, Japan.
- Montgomerie, R. F. & Rowlands, W. T.** (1937). 'Lamb Dysentery' in a foal. *Vet. Rec.* **49**, 398.
- Murrell, T. G. C., Ingham, B. G., Moss, J. R. & Taylor, W. B.** (1987). A hypothesis concerning *Clostridium perfringens* type A enterotoxin (CPE) and sudden infant death syndrome (SIDS). *Medical hypotheses*. **22**, 401-413.
- Murrell, W. G., Stewert, B., O'Neill, C., Siarakas, S. & Kariks, S.** (1993). Enterotoxigenic bacteria in SIDS. *J. Med. Micro.* **39**, 114-127.
- Myers, L. L., Shoop, D.S. & Byers, D.** (1987). Diarrhoea associated with enterotoxigenic *Bacteroides fragilis* in foals. *Am. J. Vet. Res.* **48**, 1565-1567.
- Neu, H. C.** (1988). Colonization Resistance: Fact or fancy, significant or insignificant. *Eur. J. Clin. Micro. Inf. Dis.* **7**, 91-92.
- Niilo, L.** (1980). *Clostridium perfringens* in Animal disease: A review of current knowledge. *Can. Vet. J.* **21**, 141-148.
- Niilo, L.** (1987). Toxigenic characteristics of *Clostridium perfringens* type C in enterotoxemia of domestic animals. *Can. J. Vet. Res.* **51**, 224-228.

- Niilo, L. & Chalmers, G. A.** (1982). Hemorrhagic enterotoxemia caused by *Clostridium perfringens* type C in a foal. *Can. Vet. J.* **23**, 299-301.
- Northumbria Biologicals Limited**, Cramlington, NE23 9HL.
- Oakley, C. L. & Warrack, G. H.** (1953). Routine typing of *Clostridium welchii*. *J. Hyg. Camb.* **51**, 102-107.
- Oxoid**, Unipath Limited, Wade Road, Basingstoke, Hampshire, RG24 0PW.
- Palmer, J. E.** (1985). Gastrointestinal Diseases of foals. *Vet. Clin. Nth. Am. : Eq. Pract.* **1**, 151-168.
- Pearson, E. G., Hedstrom, O. R., Sonn, R. & Wedam, J.** (1986). Hemorrhagic enteritis caused by *Clostridium perfringens* Type C in a foal. *J. Vet. Med. Assoc.* **188**, 1309.
- Perelle, S., Gibert, M., Boquet, P. & Popoff, M. R.** (1993). Characterisation of *Clostridium perfringens* Iota toxin genes and expression in *Escherichia coli*. *Infect. Immun.* **61**, 5147-5156.
- Pharmacia Biotech Limited**, Davy Avenue, P.O. Box 100, Knowlhill, Milton Keynes, MK5 8PB.
- Pons, J., Picard, B., Neil, P., Leluan, G. & Goullet, P.** (1993). Esterase electrophoretic polymorphism of human and animal strains of *Clostridium perfringens*. *Appl. Environ. Micro.* **59**, 496-501.
- Prescott, J. F. & Hoffman, A. M.** (1993). *Rhodococcus equi*. *Vet. Clin. Nth. Am. : Eq. Prac. : Update on infectious diseases.* **9**, 375-384. Edited by A. S. Turner, & J. L. Traub-Dargatz. London: W.B.Saunders Company.
- Promega Corporation**, Delta House, Enterprise Road, Chilworth Research Centre, Southampton, SO16 7NS.
- Qiagen Inc.**, 9259 Eton Avenue, Chatsworth, CA 91311, USA.
- Reed, S., Traub, J., Evermann, J., Bayly, W., Penney, R., Simon, B. & Ward, A.** (1983). Foal enteritis. *Eq. Pract.* **5**, 19-30.

- Reynolds, D. J., Morgan, J. H., Chanter, N., Jones, P. W., Bridger, J. C., Debney, T.G. & Bunch, K. J.** (1986). Microbiology of calf diarrhoea in Southern Britain. *Vet. Rec.* **119**, 34-39.
- Robins-Browne, R. M.** (1985). Pathogenesis of bacterial diarrhoea. In: *Infectious diarrhoea in the young*. pp.259-266. Edited by S. Tzipori *et al.* London: Elsevier Science Publishers.
- Rood, J. I. & Cole, S. T.** (1991). Molecular Genetics and pathogenesis of *Clostridium perfringens*. *Micro. Rev.* **55**, 621-648.
- Rothman, K. J.** (1986). Measure of effect. In: *Modern Epidemiology*. pp. 38-39. Boston, Toronto: Little Brown & Co.
- Rowley, D.** (1985). IgA antibodies and gut immunity. In: *Infectious diarrhoea in the young*. pp.419-427. Edited by S. Tzipori *et al.* London: Elsevier Science Publishers.
- Ryu, S. & Labbé, R. G.** (1993). A 48 kilodalton enterotoxin-related protein from *Clostridium perfringens* vegetative and sporulating cells. *Int. J. of Food Microbiol.* **17**, 343-348.
- Saito, M., Matsumoto, M. & Funabashi, M.** (1992). Detection of *Clostridium perfringens* enterotoxin gene by the polymerase chain reaction amplification procedure. *Int. J. Food Microbiol.* **17**, 47-55.
- Sambrook, J., Fritsch, E. F. & Maniatis, T.** (1989). Molecular cloning : A laboratory manual. 2nd. Edition. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.
- Samuel, S.C., Hancock, P. & Leigh, D. A.** (1991). An investigation into *Clostridium perfringens* enterotoxin - associated diarrhoea. *J. Hosp. Inf.* **18**, 219-230.
- Sanger, F., Nicklen, S. & Coulson, A. R.** (1977). DNA Sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* **74**, 5463-5467.
- Savage, D. C.** (1977). Microbial ecology of the gastrointestinal tract. *Ann. Rev. Micro.* **31**, 107-133.

S.E.R.C. (Statistics & Epidemiology Research Corporation) / Cytel Software

Corporation. (1985) Egret (software).

Serrano, A. M. & Schneider, I. S. (1978). New modification of Willis and Hobbs' method for identification of *Clostridium perfringens*. *J. Path. Bact.* **35**, 809-810.

Shann, F., Lawrence, G. & Jun-Di, P. (1979). Enteritis necroticans in China. *Lancet* **i**, 1083-1084.

Shanson, D. C. (1982). Microbiology in Clinical Practice. London: Wright.

Sherwood, D., Snodgrass, D. R. & Lawson, G. H. K. (1983). Prevalence of enterotoxigenic *Escherichia coli* in calves in Scotland and Northern England. *Vet. Rec.* **113**, 208-212.

Shoemaker, S. P. & Pierson, M. D. (1976). "Phoenix phenomenon" in the growth of *Clostridium perfringens*. *Appl. Environ. Micro.* **32**, 803-807.

Sebald, M. & Costilow, R. N. (1975). Minimal growth requirements for *Clostridium perfringens* and isolation of auxotrophic mutants. *Appl. Micro.* **29**, 1-6.

Sigma Chemical Company Limited, Fancy Road, Poole, Dorset, BH17 7NH.

Sims, L. D., Tzipori, S., Hazard, G. H. & Carroll, C. L. (1985). Haemorrhagic necrotising enteritis in foals associated with *Clostridium perfringens*. *Aust. Vet. J.* **62**, 194-196.

Skjelkvale, R., Stringern, M. F. & Smart, J. L. (1979). Enterotoxin production by lecithinase positive and lecithinase negative *Clostridium perfringens* isolated from food poisoning outbreaks and other sources. *J. Appl. Bact.* **47**, 329-339.

Smith, B. P., Reina-Guerra, M. & Hardy, A. J. (1978). Prevalence and epizootiology of Equine Salmonellosis. *J. Am. Vet. Med. Assoc.* **172**, 353-356.

Smith, L. D. S. & Williams, B. L. (1984). The pathogenic anaerobic bacteria. pp.113. Springfield, USA: Charles C. Thomas.

Snodgrass, D. R. (1986). Evaluation of a combined rotavirus and enterotoxigenic *Escherichia coli* vaccine in cattle. *Vet. Rec.* **119**, 39-43.

- Snodgrass, D. R. & Browning, G. F.** Characterisation of an equine rotavirus.
Equine Infectious Diseases VI (conf.), 219-223.
- Snodgrass, D. R., Terzolo, H. R., Sherwood, D., Campbell, I., Menzies, J. D. & Synge, B. A.** (1986). Aetiology of diarrhoea in young calves. *Vet. Rec.* **119**, 31-34.
- Srivastava, O. P. & Fitz-James, P. C.** (1981). Alteration by heat activation of enzymes localised in spore coats of *Bacillus cereus*. *Can. J. Bacteriol.* **27**, 408-416.
- Sterne, M. & Warrack, G. H.** (1964). The types of *Clostridium perfringens*. *J. Pathol. Bact.* **8**, 279-283.
- Stewart, G. S. A. B., Johnstone, K., Hageberg, E. & Ellar, D. J.** (1981). Commitment of bacterial spores to germinate: a measure of the trigger reaction. *Biochem. J.* **198**, 478-485.
- Strickland, K. L., Lenihan, P., O'Connor, M. G. & Condon, J. C.** (1982). Diarrhoea in foals associated with rotavirus. *Vet. Rec.* **112**, 421.
- Stringer, M. F.** (1985). *Clostridium perfringens* type A food poisoning. In: *Clostridia in gastrointestinal disease*. pp.117-143. Edited by S. P. Borriello. Boca Raton, Florida: CRC Press.
- Stringer, M. F., Turnbull, P. C. B. & Gilbert, R. J.** (1980). Application of serological typing to the investigation of outbreaks of *Clostridium perfringens* food poisoning, 1970-1978. *J. Hyg. Camb.* **84**, 443.
- Stubbings, D. P.** (1990). *Clostridium perfringens* enterotoxaemia in two young horses. *Vet. Rec.* **127**, 431.
- Sutton, R. G. A. & Hobbs, B. C.** (1968). Food poisoning caused by heat-sensitive *Clostridium welchii*. A report of five recent outbreaks. *J. Hyg.* **66**, 367-374.
- Tannock, G. W.** (1983). Control of Gastrointestinal pathogens by normal flora. *Biological Control* **12**, 374-382.
- Titball, R. W., Hunter, S. E., Martin, K. L., Morris, B. C., Shuttleworth, A. D., Rubidge, T., Anderson, D. W. & Kelly, D. C.** (1989). Molecular cloning and

- nucleotide sequence of the alpha-toxin (phospholipase C) of *Clostridium perfringens*. *Infect. Immun.* **57**, 367-376.
- Traub-Dargatz, J. L., Gay, C. C., Evermann, J. F., Ward, A. C. S., Zeglen, M. E., Gallina, A.M. & Salman M. D.** (1988). Epidemiologic survey of diarrhoea in foals. *J. Am. Vet. Med. Assoc.* **192**, 1553-1556.
- Tsai, C. C. & Riemann, H. P.** (1974). Relation of enterotoxigenic *Clostridium perfringens* type A to food poisoning. I. Effect of heat activation on the germination, sporulation and enterotoxigenesis of *Clostridium perfringens*. *Taiwan I. Hsueh. Hui. Tsa. Chih.* **73**, 653-659.
- Tschivdewahn, B., Notermans, S., Wernars, K. & Untermann, F.** (1991). The presence of enterotoxigenic *Clostridium perfringens* strains in faeces of various animals. *Int. J. Food Biochem.* **14**, 175-178.
- Tzipori, S.** (1985a). A comparative study on important pathogens causing diarrhoea in calves and piglets. In: *Infectious diarrhoea in the young*. Edited by S. Tzipori, et al.. pp. 371-379. Elsevier Science Publishers.
- Tzipori, S.** (1985b). The relative importance of enteric pathogens affecting neonates of domestic animals. *Adv. Vet. Sci. Comp. Med.* **29**, 103-206.
- Tzipori, S.** (1985c). Cryptosporidiosis: infection and disease. In: *Infectious diarrhoea in the young*. Edited by S. Tzipori, et al.. pp. 327-334. Elsevier Science Publishers.
- Tzipori, S., Hayes, J., Sims, L. & Withers, M.** (1984). Streptococcus durans: an unexpected enteropathogen of foals. *J. Inf. Dis.* **150**, 589-593.
- United States Biochemical**, P.O. Box 22400, Cleveland, Ohio 44122, USA.
- Urquhart, K.** (1981). Diarrhoea in foals. *Eq. Pract. -In Practice. -Vet. Rec. Suppl.* **3**, 22-29.
- Van Baelan, D. & Devriese, L. A.** (1987). Presence of *Clostridium perfringens* enterotoxin in intestinal samples from farm animals with diarrhoea of unknown origin. *J. Vet. Med.* **34**, 713-716.

- Van Damme-Jongsten, M., Haagsma, J. & Notermans, S.** (1990a). Testing strains of *Clostridium perfringens* type A isolated from diarrhoeic piglets for the presence of the enterotoxin gene. *Vet. Rec.* **126**, 191-192.
- Van Damme-Jongsten, M., Rodhouse, J., Gilbert, R. J. & Notermans, S.** (1990b). Synthetic DNA probes for detection of enterotoxigenic *Clostridium perfringens* strains isolated from outbreaks of food poisoning. *J. Clin. Micro.* **28**, 131-133.
- Van Damme-Jongsten, M., Wernars, K. & Notermans, S.** (1989). Cloning and sequencing of the *Clostridium perfringens* enterotoxin gene. *Antonie van Leeuwenhoek* **56**, 181-190.
- Van der Waaij, D.** (1987). Antibiotics, Anaerobes and colonization resistance. In: *Recent advances in anaerobic bacteriology*. pp.100-107. Edited by S.P. Borriello & J. M. Hardie. Martinus Nijhoff.
- Van der Waaij, D.** (1988). Evidence of immunoregulation of the composition of intestinal microflora and its practical consequences. *Eur. J. Clin. Micro. Infect. Dis.* **7**, 103-106.
- Van der Waaij, D.** (1989). The ecology of the human intestine and its consequences for overgrowth by pathogens such as *Clostridium difficile*. *Ann. Rev. Micro.* **43**, 69-87.
- Van Kessel, L. J. P., Verbrugh, H. A., Stringer, M. F. & Hoekstra, J. B. L.** (1985). Necrotizing enteritis associated with toxigenic type A *Clostridium perfringens*. *J. Inf. Dis.* **151**, 974.
- Vary, J. C.** (1978). Glucose - initiates germination in *B. megaterium* spores. In: *Spores VII*. pp.104-108. Edited by G. Chambliss & J. C. Vary. Washington DC: American Society for Microbiology.
- Walker, P. D.** (1985). Pig-Bel. In: *Clostridia in gastrointestinal disease*. Edited by S. P. Borriello. pp. 93-115. Boca Raton, Florida: CRC Press.
- Walker, P. D.** (1992). Bacterial vaccines: old and new, veterinary and medical. *Vaccine*. **10**, 977-990.

- Walter, S. D. & Cook, R. J.** (1991). A comparison of several point estimators of the odds ratio in a single 2 x 2 contingency table. *Biometrics* **47**, 795-811.
- Whatman International Limited**, St. Leonard's Road, 20 / 20 Maidstone, Kent, ME16 0LS.
- Whitlock, R. H.** (1975). Acute diarrhoeal disease in the horse. *Proc. Am. Ass. Eq. Pract.* **21**, 390-401.
- Wierup, M. & DiPietro, J. A.** (1981). Bacteriologic examination of equine faecal flora as a diagnostic tool for equine intestinal clostridiosis. *Am. J. Vet. Res.* **42**, 2167.
- Wilkinson, M.** (1992). *Clostridium perfringens* as an agent of infectious diarrhoea. *Dissertation: pg D/MSc in Biomedical Sciences*. Portsmouth University.
- Williams, R., Piper, M., Borriello, P., Barclay, F., Welch, A., Seal, D. & Sullens, K.** (1985). Diarrhoea due to enterotoxigenic *Clostridium perfringens*: Clinical features and management of a cluster of ten cases. *Age and Ageing* **14**, 296-302.
- Williams-Smith, H.** (1965). The development of the alimentary tract in young animals. *J. Path. Bact.* **90**, 495-513.
- Williams-Smith, H. & Crabb, W. E.** (1961). The faecal bacterial flora of animals and man: its development in the young. *J. Path. Bact.*, **82**, 53-66.
- Wnek, A. P. & McClane, B. A.** (1989). Preliminary evidence that *Clostridium perfringens* type A enterotoxin is present in a 160,000-Mr complex in mammalian membranes. *Infect. Immun.* **57**, 574-581.
- Woolf, B.** (1955). On estimating the relation between blood group and disease. *Annal. Hum. Gene.* **19**, 251-253.
- Xiao, L. & Herd, R. P.** (1994). Epidemiology of equine *Cryptosporidium* and *Giardia* infections. *Eq. Vet. J.* **26**, 14-17.
- Yan, W. K.** (1989). Use of host modified bacteriophages in development of a phage typing scheme for *Clostridium perfringens*. *Med. Lab. Sci.* **46**, 186-193.

Yamagoshi, T., Serikawa, T., Morita, R., Nakamura, S. & Nishida, S. (1976).

Persistent high numbers of *Clostridium perfringens* in the intestines of Japanese adults. *Japan. J. Micro.* **20**, 397-403.

Yasuda, Y. & Tochikubo, K. (1984). Effect of glucose on the interaction of

hydrophobic compounds with the alanine receptor field of *Bacillus subtilis* spores during initiation of germination. *Microbiol. Immunol.* **28**, 1202-1210.

Zeissler, J. & Rassfeld-Sterberg, C. (1949). Enteritis necroticans due to

Clostridium welchii type F. *Br. Med. J.* **1**, 267-269.

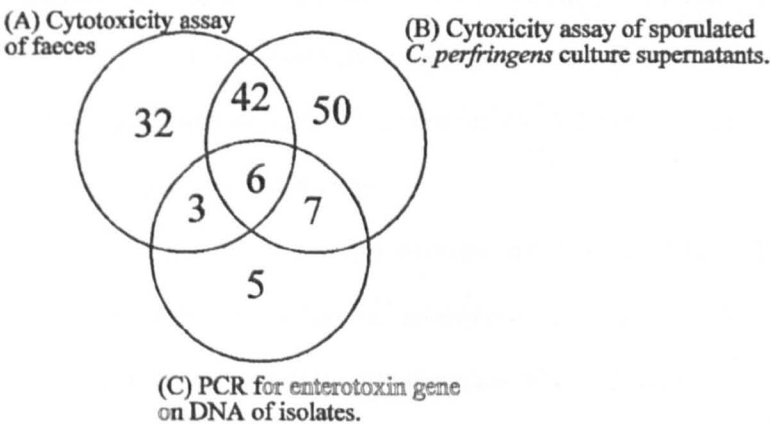
Addendum 1 (to page 96)

Detection of enterotoxin by vero-cell cytotoxicity neutralisation assay directly in faeces and in sporulated culture supernatant fractions of isolates of <i>Clostridium perfringens</i> .*								
Enterotoxin detection	Number of foals tested	Percentage of all foals positive for enterotoxin	Percentage of all foals with diarrhoea positive for enterotoxin (n = 421)	Percentage of healthy foals in-contact with diarrhoea positive for enterotoxin (n = 102)	Percentage of healthy foals not-in-contact with diarrhoea positive for enterotoxin (n = 120)	Odds Ratio	p-value	95% confidence limits
Enterotoxin detected in the faeces	643	21	19 (15) ^a	29 (12) ^a	18 (11) ^a	1.09 (1.45) ^a	0.736 (0.251) ^a	0.63 - 1.91 (0.75 - 2.98) ^a
Enterotoxigenic <i>C. perfringens</i> isolates	643	22	29	21	18	1.57	0.089	0.91 - 2.73
*analysis conducted by comparison with healthy not in contact foals by univariate analysis.								
^a percentage of samples with a cytotoxic titre great enough to detect neutralisation and which did not give a four-fold reduction in titre in the presence of enterotoxin anti-serum.								

Although enterotoxin was detectable by the presence of verocytotoxicity neutralisable by antiserum (given that the antiserum is specific), there was no relationship between detection and scouring in foals. Verocytotoxicity which could not be neutralised by enterotoxin was not associated with diarrhoea.

Addendum 2 (to page 109)

Relationship between the results for the detection of enterotoxin by three different methods (number of foals positive by 1, 2, or all 3 methods) in scouring foals.
(There were too few enterotoxin gene PCR positive isolates to complete this analysis for healthy foals.)



Measure of association between each of the different enterotoxigenicity detection methods.			
Methods	Odds Ratio	p - value	Confidence Interval
A and B	6.8	<0.001	3.88 - 11.76
A and C	3.3	0.006	1.18 - 8.87
B and C	5.4	<0.001	2.01 - 15.56

Addendum 3 (to page 123)

There was no association between the detection of verotoxicity neutralisable by commercially available antiserum to enterotoxin and diarrhoea in foals (Addendum 1). This raised several points for discussion. The intrinsic toxicity of equine faeces meant that the sample had to be diluted 1/100 before detecting enterotoxin. Furthermore, the criterion of detection was a four-fold neutralisation by antiserum. This meant that there would have to be a titre at least of 1/400 before enterotoxin could be specifically identified. It is possible that the amounts of enterotoxin that might be present in the faeces of a foal suffering diarrhoea, if indeed enterotoxigenic *C.perfringens* causes foal diarrhoea, might be somewhat lower than this. Consequently, the test would be too insensitive to reveal an association with diarrhoea.

The cytotoxicity assay of culture supernatants used much lower dilutions than used for faeces. However, for this assay production of enterotoxin is dependent on sporulation in culture. Failure of some isolates to sporulate properly may have reduced the association of positives with diarrhoea.

Another possibility is that enterotoxigenic *C.perfringens* may have sporulated in transit in spite of efforts to reduce this delay before conducting laboratory tests. This might have the effect of blurring an association of enterotoxin present in faeces with diarrhoea.

However, the PCR test detected the enterotoxin gene only in one of the healthy in contact animals, and not at all from healthy not in contact animals, and yet neutralisable verotoxicity was detected in both the faeces and supernatants of isolates from 18% of these animals (see Addendum 1).

This suggested that either the PCR test was missing some enterotoxigenic isolates, perhaps because some produce PCR inhibitors, or that the cytotoxicity assay and antiserum were not specific for enterotoxin. Comparison of results for tests of

neutralisable cytotoxicity with the enterotoxin PCR showed that there was a significant association between all three methods for scouring foals (see Addendum 2). However, the association was not very close since there were many more cytotoxin positive strains negative by PCR than PCR positives negative by one or other of the cytotoxicity tests. Nonetheless, it was the PCR positive isolates that were strongly associated with diarrhoea (see Table 23) whereas, as already mentioned, cytotoxicity in faeces or culture supernatants was not (Addendum 1).

Recently, the same samples of DNA used for the enterotoxin PCR were PCR tested using primers for the alpha toxin (data not included here). More than 95% of isolates were positive for the alpha toxin suggesting that PCR inhibitors probably could not explain the relatively low enterotoxin gene detection rate compared with rates of cytotoxicity. This strongly indicated that the isolates positive by cytotoxicity assay which were negative by the enterotoxin PCR did not represent detections of enterotoxin.

Another possibility which requires investigation is that there is another toxin antigenically related to the enterotoxin which has DNA sequence differences with the published enterotoxin gene and hence might be negative in the PCR test because of imperfect priming. If true, this might explain some of the isolates positive by the cytotoxicity test which are negative by the PCR test.

C.perfringens may produce other toxins potentially capable of verotoxicity which were neutralised by the antiserum to enterotoxin. These toxins either may have contaminated the enterotoxin preparation used for immunisation or the immunised animal may already have had antibodies to them. A subjective assessment of the characteristics of cytopathic effect and of whether they were more probably due to enterotoxin than other toxins was not made. Consequently, just as with the RPLA (see below; page 123-124), there is a question over the specificity of the antiserum to enterotoxin and its potential to cause false positives and that until specific antisera

become available, it might be prudent to add the enterotoxin PCR to the armoury of tests used to assess enterotoxigenicity.

One way forward towards a specific antiserum to the enterotoxin, other than through monoclonal antibodies, may be provided by the cloned enterotoxin gene. In its cloned form it is separated from other clostridial toxins and the host *E.coli* is not cytotoxic for Vero cells. Consequently, immunisation with purified toxin, or indeed crude toxin, from the recombinant should stimulate antibodies able to neutralise the enterotoxin and not other clostridial toxins. Additionally, selection of rabbits before immunisation on the basis of the lack of ability of their sera to neutralise the cytotoxicity of culture supernatants from a variety of isolates used in this study, should help to eliminate the possibility of natural immunity to "other toxic factors" as a cause of non-specific neutralisation. Comparison of the neutralisation activity of an antiserum produced as described above and pre-immune serum with the commercially available antiserum should help to address the question of whether or not there is an antigenically related cytotoxin produced by *C.perfringens* isolates or from another source in faeces.